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EDITORIAL

Current understanding of the functional roles of aberrantly expressed microRNAs in esophageal cancer

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Abstract

The incidence of esophageal cancer is rising, mostly because the increasing incidence of esophageal adenocarcinoma in Western countries. Despite improvements in diagnosis and treatment, the overall 5-year survival rates remain low. MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate the expression of target genes. Recently, disease specific miRNAs have been identified, which act as tumor suppressors or oncogenes. In this review, we will summarize the current knowledge about the function of aberrantly expressed miRNAs in esophageal cancer. We selected 5 miRNAs (miRNA-21, -143, -145, -196a and let-7) based on the available literature, and described their potential role in regulating pathways that are deregulated in esophageal cancer. Finally we will highlight the current achievements of using and targeting miRNAs. Because these miRNAs likely have important regulatory roles in cancer development, they open a therapeutic window for new treatment modalities.

Key words: Esophageal cancer; Esophageal squamous cell carcinoma; Esophageal adenocarcinoma; MicroRNAs; Target genes

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Core tip: MicroRNAs (miRNAs) likely have important regulatory roles in the development of cancer as they target essential pathways. Here, we review the function of aberrantly expressed miRNAs in esophageal cancer, with the aim to provide guidelines for future studies focusing on the function of miRNAs and the development of new treatment modalities.

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INTRODUCTION

Esophageal cancer is the 8th most common cancer and the 6th most lethal cancer worldwide[1]. Esophageal cancer is divided into two main histopathological subtypes; esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC develops in esophageal squamous cells while EAC develops in intestinal type epithelium containing goblet cells, which is also known as Barrett's esophagus (BE). Both cancer types are different in development, etiology and treatment. Due to fact that most patients have already distant metastases at the time of diagnosis, the prognosis of esophageal cancer remains poor. Despite improvements in diagnosis and treatment, the overall 5-year survival rate is 15%-20%, and only after treatment with a curative intent, the survival rate increases to 47%[2].

MicroRNAs (miRNAs) are small non-coding RNA molecules of 20-24 nucleotides long that modulate the expression of target genes. They are first transcribed from DNA as parts of longer molecules (pre-miRNA) and undergo final processing by dicer in the cytoplasm to form mature miRNAs[3]. miRNAs are present in tissue, blood and other body fluids and have emerged as critical components of complex functional pathways involved in processes such as differentiation, apoptosis and proliferation[4]. Recently, numerous studies have identified tissue or disease specific miRNAs by extensive miRNA-profiling. These studies have identified miRNAs which are aberrantly expressed in esophageal cancer for example miR-21, -145, -192, which are upregulated and miRNA-31, -203, -205 and let-7 which are often downregulated compared to normal esophageal tissue[5,6]. These findings have led to the identification of miRNAs that act as tumor suppressors or oncogenes. In addition, several studies have shown that aberrant expression of specific miRNAs correlates with the survival of patients with esophageal cancer, presence of metastasis and response to neo-adjuvant therapies[5]. However, to date little is known about the cellular function of these differently expressed miRNAs.

In this review, we will summarize the function of miRNAs in the carcinogenic process of the esophagus. Using PubMed, we identified studies that evaluated the effects of miRNAs in ESCC or EAC and selected five miRNAs, miRNA-21, -143, -145, -196a and let-7, which are known to be up- or downregulated in esophageal cancer. Moreover, we will describe the current development of novel anti-cancer therapy by targeting miRNAs.

MIRNA-21 ACTS AS AN ONCOGENE IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

MiRNA-21 is reported to be an oncogene and is highly expressed in various malignancies[6]. Next to colon and gastric cancer[7], miRNA-21 expression is also upregulated in esophageal cancer. In ESCC, miRNA-21 expression is consistently reported to be higher expressed compared to normal adjacent squamous epithelium[8-10]. Moreover, increased expression of miRNA-21 is associated with more advanced stages of ESCC[8,9]. Profiling studies of EAC have also shown upregulated miRNA-21 expression in EAC compared to normal adjacent squamous epithelium[11-13]. In addition, high miRNA-21 expression is already observed in the known precursor of EAC, BE[12,14,15]. This indicates that miRNA-21 plays an important role in the carcinogenic process that occurs in the esophagus. Several studies have focused on functional roles of miRNA-21 during cancer development of the esophagus.

In vitro studies using ESCC cell lines showed increased cell viability upon miRNA-21 precursor transfection[8,9]. This increase in cell viability could be the effect of miRNA-21 targeting the PI3K pathway. This pathway regulates various cellular processes including metabolism, proliferation and cell migration and is one of the most critical cancer-promoting pathways. In short, PI3K catalyzes the formation of PIP3, which transduces activating signals to the serine-threonine kinase AKT, which in his turn is able to phosphorylate a wide array of additional substrates that also induces proliferation and survival. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is an antagonist of this pathway; it dephosphorylates PIP3 and subsequently inhibits activation of AKT[16]. Dysregulation of the PI3K pathway through selective mutations have been reported in numerous cancers[16].

In ESCC, Ma *et al*[9] observed an inverse correlation, however not statistically significant, between miRNA-21 expression and PTEN protein expression (Table 1 summarizes the function of all miRNAs described in this review). *In vitro* transfection experiments overexpressing miRNA-21 showed no significant effect on PTEN mRNA expression, but a downregulation of PTEN protein expression was observed. This suggests that miRNA-21 targets PTEN at a post-transcriptional level. In addition, knockdown of miRNA-21 leads to significantly upregulated PTEN expression[9,17,18]. Moreover, Huang *et al*[17] showed, that after PTEN downregulation, pAKT is dephosphorylated. Based on these studies, it can be concluded that miRNA-21 inhibits PTEN thereby inhibiting the PI3K pathway leading to increased proliferation and cancer cell survival.

Next to regulating the PI3K pathway, Liu *et al*[10] reported that miRNA-21 targets programmed cell death 4 (PDCD4) gene in ESCC. This is in line with other studies, which have shown that miRNA-21 directly targets PDCD4 in colorectal cancer, hepatocellular carcinoma and breast cancer[19-21]. PDCD4 is a recently discovered tumor suppressor which controls cell migration, directs apoptosis and regulates the

Table 1 MiRNAs and their targets in esophageal cancer

miRNA	Expression profile	ESCC	EAC	Related biological function	Ref.
miRNA-21	↑	PTEN		Cell proliferation	[8,17,18]
		PDCD4		Cell migration, apoptosis	[9]
Let-7	↓	HMGA2		Cell proliferation	[25,26]
		IL-6		Cytokine activity	[27]
miRNA-143 and -145	↓	FSCN	FSCN	Cell migration	[29,32]
		ERK5		Cell proliferation, cell migration	[30]
miRNA-196a	↑		Annexin A1	Apoptosis	[38,39]
			SPRR2C	Pseudogene	[38]
			S100A9	Cell differentiation, cell proliferation	[38]
			KRT5	Cell architecture	[38]
		RAP1A		Cell proliferation, cell adhesion and cell migration	[41]

miRNAs with corresponding expression profile (↑ = upregulated, ↓ = downregulated in esophageal cancer), described targets and related biological function in esophageal squamous cells carcinoma (ESCC) and esophageal adenocarcinoma (EAC).

cellular response to DNA damage. In various types of cancer tissue, PDCD4 expression is downregulated. This was also observed in ESCC, where high expression of PDCD4 was detected in normal squamous epithelium in contrast to ESCC[10,22]. In addition, in ESCC an inverse correlation of PDCD4 with miRNA-21 was reported[10]. Future studies should determine if miRNA-21 also targets PDCD4 during the carcinogenic processes that occur in the transition from BE to EAC.

LET-7 ACTS AS A TUMOR SUPPRESSOR IN ESOPHAGEAL CANCER

One of the first miRNAs discovered, the let-7 family is comprised of 12 family members and have an overlapping set of target genes[23]. In the earliest phases of embryology, the let-7 family is not expressed while expression is upregulated during later stages of development. During neoplastic progression, let-7 is often downregulated and therefore considered to be a tumor suppressor[24]. In both ESCC and EAC, downregulated expression of let-7 has been reported and downregulated expression is associated with a poor prognosis[5,6].

Overexpression of let-7 in an ESCC cell line resulted in decreased cell viability compared to cells transfected with a let-7 inhibitor[25]. Several studies have identified high mobility group AT-Hook (HMGA2) as a putative target of let-7[24]. In contrast to let-7, expression of HMGA2 is prominent during development and absent in adult human tissues. However, during cancer development HMGA2 is re-expressed and acts as an oncogene by regulating cell proliferation[24]. An *in vitro* study of Liu *et al*[25] observed decreased HMGA2 protein expression after let-7 overexpression in an ESCC cell line. However, no difference was observed in mRNA expression among the different groups suggesting that let-7 targets HMGA2 at a post-transcriptional level. Another paper from the same group confirmed with a luciferase assay that let-7 directly targets HMGA2 in the esophagus[26]. To our knowledge, no studies regarding HMGA2 expression have been performed in BE or EAC. It would be interesting to determine if downregulated expression of let-7 also results in upregulation of HGMA2 in EAC. Future studies are required to elucidate this in detail.

Besides HGMA2 as putative target of let-7, Sugimura *et al*[27] reported an inverse association between both let-7b/c and IL6 expression in ESCC tissue. IL-6 was identified as potential target using the Target scan database. In addition, *in vitro* studies have shown that let-7c overexpression significantly reduced IL-6 expression in ESCC cell lines. Moreover, expression of STAT3, a downstream target of IL-6, was also reduced after let-7c overexpression[27]. Findings by Sung *et al*[28] supported the results of this study as they showed that let-7 directly targets IL6 in cancer associated mesenchymal stem cells involved in prostate cancer.

miRNA-143 AND miRNA-145 JOINTLY ACT AS TUMOR SUPPRESSORS

miRNA-143 and miRNA-145 are positioned in close proximity with each other on chromosome 5 and this suggests that they have similar biological functions. The expression of miRNA-143 and miRNA-145 is down-regulated in both ESCC and EAC[29-31]. Furthermore, downregulation of these miRNAs is reported to be associated with increased invasion depth and lymph node metastasis[29,30] suggesting a tumor suppressive role for these miRNAs. *In vitro* studies have shown that overexpression of both miR-143 and miR-145 significantly reduced cell proliferation and migration while apoptosis was increased[30-33]. In addition, overexpression of miRNA-143 decreased invasive properties of ESCC cell lines[30].

Two different groups have shown that miRNA-145 directly targets fascin homolog (FSCN). Following overexpression of miRNA-145, a significant down-regulation in FSCN expression was observed[29,32]. In addition, Liu *et al*[29] showed that miRNA-143

also directly targets FSCN, with even a 2-fold higher inhibition efficacy compared to miRNA-145. FSCN is an important regulatory element in the maintenance and stability of filamentous actin. It organizes filamentous actin in well-ordered parallel bundles and plays a crucial role in the formation of membrane protrusions. It has been suggested that FSCN has an oncogenic role as it promotes cell motility and migration[29,32]. Knockdown of FSCN in an ESCC cell line resulted in decreased cell growth and cell invasion[32]. In addition, high expression of FSCN correlates with poor survival in ESCC[34].

Besides the effect on FSCN expression, it was also suggested that miRNA-143 targets extracellular-signal-regulated kinase 5 (ERK5) expression. This kinase is a member of the mitogen activated protein kinase family and is important for cell proliferation and angiogenesis. Some reports have shown a potential role for ERK5 in cancer progression[35]. To our knowledge, only one study investigated ERK5 expression in ESCC on protein level. After overexpression of miRNA-143 in an ESCC cell line, ERK5 expression was downregulated[30]. In bladder cancer, overexpression of both miRNA-143 and -145 reduced ERK5 expression[36]. However, further studies are necessary to evaluate if ERK5 is a direct target of miRNA-143 and -145 and if the downregulation is a downstream effect of targeting another gene in the MEK5/ERK5 pathway in the esophagus.

MIRNA-196a ACTS AS AN ONCOGENE

The miRNA-196 family contains three miRNAs: miRNA-196a-1, miRNA-196a-2 and miRNA196b. miRNA-196a-1 is located on chromosome 17 while miRNA-196a-2 is located on chromosome 12. When these miRNAs are processed to mature miRNAs, both have identical mature nucleotide sequences (miRNA196a)[37]. In BE, the precursor of EAC, the expression of miRNA-196a is already significantly upregulated compared to the normal squamous epithelium. This increased expression is also observed in EAC[38]. Further studies are needed to specifically identify whether this is miRNA-196a-1 or -196a-2 that is upregulated in BE and EAC. For ESCC, publications concerning miRNA-196 expression are limited.

Functional assays have shown that overexpression of miR-196a in EAC cells resulted in increased cell proliferation compared to control cells[39]. However, this study used SEG-1 cells, which later found to be a cell line derived from lung cancer[40]. The first study which evaluated the effect of increased miRNA-196a expression in EAC found an inverse correlation comparing the expression of Annexin A1 and miRNA-196a. As expected, miRNA-196a was upregulated while Annexin A1 was downregulated in EAC compared to normal squamous epithelium[38]. Annexin A1 is a crucial factor regulating apoptosis and suppression of Annexin is often reported in malignancies[39]. To confirm that annexin A1 was a direct target of miRNA-196a, luciferase assays were performed, showing that miRNA-196a directly targets annexin A1[39]. The same group also showed an inverse correlation between the expression of miRNA-196a and SPRR2C, S100A9 and Keratin 5. To confirm these putative targets, *in vitro* miRNA-196a overexpression and luciferase-based assays were performed in EAC cell lines[38].

In addition, Wang *et al*[41] showed in ESCC cell lines that miRNA-196a directly inhibits RAP1A. RAP1A belongs to the family of RAS-related proteins regulating a wide range of biological processes*, i.e.,* cell proliferation, cell adhesion and cell mobility[41]. In patients with ESCC, high expression of RAP1a is associated with lymph node metastasis. Overexpression of RAP1A in an ESCC cell line resulted in increased migration and invasion. In addition knockdown of RAP1A resulted in decreased migration and invasion[41]. To our knowledge, this is the only study which have reported RAP1A as a target for miRNA-196a. It would be interesting if other groups can confirm these results and to further evaluate the effect of reduced expression of RAP1A in ESCC.

CLINICAL APPLICATIONS OF MIRNAS

Results of the studies summarized above highlight the important regulatory roles of miRNAs in mediating changes in gene expression during the development of esophageal cancer. These miRNAs are excellent candidates for the development of novel treatment modalities. Depending on the oncogenic or tumor suppressive role(s) of the specific miRNA, it may be possible to inhibit or replace its function through the use of miRNA mimics and inhibitors. However until now, the research field has mainly focused on the identification of down-stream targets of miRNAs using *in vitro* studies (Figure 1). Animal studies should be the next step in order to elucidate whether the manipulation of specific miRNAs could provide a new therapeutic window for esophageal cancer. In a mouse model of ESCC, knockdown of miRNA-21 reduced tumor size and weight, suggesting a potential role for miRNA-21 as therapeutic target in the treatment of ESCC[9]. This study indicates that it should be possible to translate the *in vitro* results to animal studies.

Recently, the first human study for the evaluation of miRNA-based therapy was published. In patients with chronic hepatitis C virus (HCV), Miravirsen, an antisense oligonucleotide which binds and thereby blocks miRNA-122, was randomly compared with placebo. Treatment with Miravirsen was found to dose-dependently reduce HCV RNA levels compared to placebo[42]. In addition, MRX34, a mimic of the tumor suppressor miRNA-34, is currently evaluated in an open-label phase 1 clinical trial in patients with unresectable liver cancer (ClinicalTrials.gov number: NCT01829971). These crucial studies will pave the way for other phase 1 studies for miRNA-based therapies.

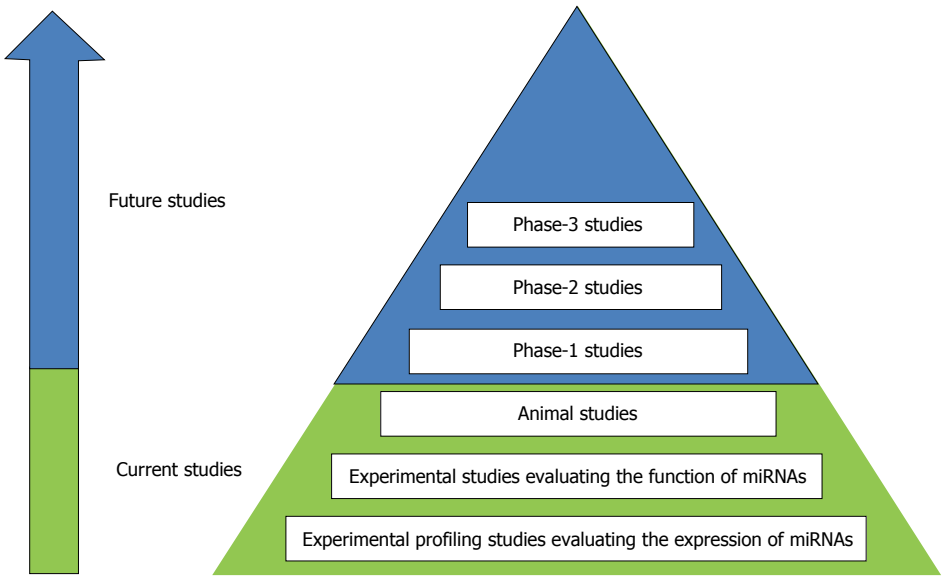

Figure 1 Overview of study phases for developing miRNA-based therapies for esophageal cancer. An overview of the current and future studies for developing miRNA-based therapies. The research field of esophageal cancer has mainly focused on the identification of miRNAs (profiling studies) and evaluating their specific functions by identifying the targets using *in vitro* experiments. Animal studies provide insight whether the manipulation of miRNAs provide new therapeutic windows. For the future, phase-1, -2 and -3 studies should be conducted in order to determine whether the manipulation of miRNAs could provide a new therapeutic window for esophageal cancer.

Moreover the expression of specific miRNAs could be used in order to individualize the treatment for patients with esophageal cancer. For example, a low expression of let-7c was found to correlate with a poor response to chemotherapy[27]. Sensitivity to cisplatin, which is commonly used as chemotherapy in esophageal cancer, increased after transfection with let-7c in ESCC cell lines[27]. Furthermore, Hummel *et al*[43] compared the miRNA signature of chemoresistant esophageal cell lines (both EAC as ESCC) to chemotherapy sensitive controls and identified 18 miRNAs that were significantly dysregulated compared to controls. Q-RT-PCR validation of the microarray experiments has identified numerous miRNAs aberrantly expressed in cisplatin resistant esophageal cancer cell lines and 5-FU resistant esophageal cancer cell lines It would be of high interest to further investigate if these results can be translated into a clinical setting in order to modify the treatment for patients with esophageal cancer and increase disease survival.

CONCLUSION

This review describes the current knowledge regarding the function of aberrantly expressed miRNAs in esophageal cancer. Overall there is a limited number of studies published evaluating the effect of miRNAs in esophageal cancer; however, the knowledge in this field is rapidly expanding. Further studies to identify putative targets of miRNAs will improve our understanding of their function in the development and progression of esophageal cancer. This knowledge will improve the ability to utilize miRNAs clinically as therapeutic targets and/or as prognostic markers.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

Optimal management for alcoholic liver disease: Conventional medications, natural therapy or combination?

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Abstract

Alcohol consumption is the principal factor in the pathogenesis of chronic liver diseases. Alcoholic liver disease (ALD) is defined by histological lesions on the liver that can range from simple hepatic steatosis to more advanced stages such as alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure. As one of the oldest forms of liver injury known to humans, ALD is still a leading cause of liver-related morbidity and mortality and the burden is exerting on medical systems with hospitalization and management costs rising constantly worldwide. Although the biological mechanisms, including increasing of acetaldehyde, oxidative stress with induction of cytochrome p450 2E1, inflammatory cytokine release, abnormal lipid metabolism and induction of hepatocyte apoptosis, by which chronic alcohol consumption triggers serious complex progression of ALD is well established, there is no universally accepted therapy to prevent or reverse. In this article, we have briefly reviewed the pathogenesis of ALD and the molecular targets for development of novel therapies. This review is focused on current therapeutic strategies for ALD, including lifestyle modification with nutrition supplements, available pharmacological drugs and new agents that are under development, liver transplantation, application of complementary medicines, and their combination. The relevant molecular mechanisms of each conventional medication and natural agent have been reviewed according to current available knowledge in the literature. We also summarized efficacy *vs* safety on conventional and herbal medicines which are specifically used for the prevention and treatment of ALD. Through a system review, this article highlighted that the combination of pharmaceutical drugs with naturally occurring agents may offer an optimal management for ALD and its complications. It is worthwhile to conduct large-scale, multiple centre clinical trials to further prove the safety and benefits for the integrative therapy on ALD.

Key words: Alcoholic liver disease; Alcohol hepatitis;

Conventional medicines; Natural medicines; Hepatic lipid metabolism; Hepatic inflammation; Combination therapy

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Core tip: The aim of this article is to review the impairment of hepatocellular dysfunction in alcoholic liver diseases and their prospective managements. Specifically, we focused on the natural therapies with their efficacies and safeties. Moreover, we summarized molecular mechanisms of herbal therapy to treat alcoholic liver disease (ALD). With evidence-based natural therapy, this article highlighted that the combination of pharmaceutical drugs with naturally occurring agents may offer an optimal management for this complex liver disease. It is worthwhile to conduct large-scale, multiple centre clinical trials further to prove the safety and benefits for the integrative therapy on ALD.

Kim MS, Ong M, Qu X. Optimal management for alcoholic liver disease: Conventional medications, natural therapy or combination? *World J Gastroenterol* 2016; 22(1): 8-23 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i1/8.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i1.8

INTRODUCTION

Alcohol is a psychoactive substance and has been widely used in many cultures for centuries. Alcoholic abuse causes a large range of diseases and is also a social and economic burden in world societies. Reduction of alcohol consumption is now becoming the global strategy, with attempts to define "harmful use" of alcohol by the World Health Organization (WHO) to reduce the associated morbidity and mortality. Alcohol intake is a principal etiological factor in chronic liver diseases. Alcoholic liver disease (ALD), which initially manifests as hepatic fat accumulation, followed by an inflammatory response to induce final stage liver failure, is now a deleterious health problem[1]. Alcohol abuse accelerates various types of liver diseases, such as alcoholic fatty liver disease (AFLD), alcoholic steatohepatitis, alcoholic hepatitis (AH), progressive fibrosis, liver cirrhosis and liver failure[2]. Patients with ALD, especially with alcoholic cirrhosis, are associated with increased risk of hepatocellular carcinoma (HCC)[3,4].

The prevalence of ALD has increased in the last years, parallel with the increasing alcohol consumption in the western world as well as in Asian counties[5]. According to the WHO report in 2011, chronic alcohol consumption results in approximately 2.5 million deaths each year with much of the burden related to ALD[6]. Though ALD significantly contributes to the rising morbidity and mortality statistics and related health expenses, there is a lack of effective treatments for ALD, especially cirrhosis and HCC. Abstention from alcohol may reverse the early stage of ALD to a normal condition. The treatment for ALD with conventional medicines, mainly pharmaceutical medications, has limited success with side-effects. Recently, natural medicines, which mainly apply herb-derived agents, are emphasized as alternative therapies to manage the various alcohol-related liver diseases. It is the aim of this article to provide an overview of understanding the mechanisms of ALD, which could generate therapeutic interventions with conventional medicines, natural therapies and their combinations to reverse or retard the progression of ALD.

Pathogenesis of ALD is multifactorial. Strategically, the liver is the most important organ to target as about 90% of alcohol intake is metabolized by the liver[7]. Alcohol consumption can cause fatty liver by disrupting hepatic lipids and glucose metabolism. Furthermore, ethanol and its oxidative and non-oxidative metabolites have direct toxic effects on the liver[8]. Histologically, fatty liver, or HS, is a malfunctioned fat accumulation in the parenchymal cells of the liver. HS is a reversible lesion. Fibrosis (or scar formation) is the subsequent result if liver injury persists. Fibrosis determined by biopsy increases the likelihood of progression to cirrhosis and end-stage liver disease. Biochemically, alcohol abuse contributes to a serious complex phenomenon involving different molecular and biological mechanism to induce ALD. Many hypotheses have been investigated and established to explain the pathogenic mechanisms of ALD. These include: (1) activation of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) to cause the over generation of acetate[9]; (2) induction of cytochrome P450 2E1 (CYP2E1) for alcoholic oxidative stress and hepatotoxicity[10]; (3) abnormal lipid metabolism by increasing fatty acid (FA) synthesis and decreasing of FA oxidation[10]; (4) hepatic inflammation indicated by an increase of tumour necrosis factor-α (TNF-α) and cytokine release[11]; (5) induction of hepatocyte apoptosis and subsequent activation of Kupffer cells; (6) increased hepatic levels of cellular fibronectin and tissue growth factor-β (TGF-β) related to the activation of hepatic stellate cells[12]; and (7) ethanol-induction of gut endotoxins[13,14]. Proposed mechanisms impacting hepatocellular dysfunction by alcohol exposure within ALD pathogenesis are summarized in Figure 1. To further improve current strategies for managing ALD, a deeper understanding of the pathomechanisms of the disease is needed. Various curative approaches based on these mechanisms could prevent the progression of ALD and its downstream sequelae.

CURRENT MANAGEMENT FOR ALD

General management of ALD should initially be

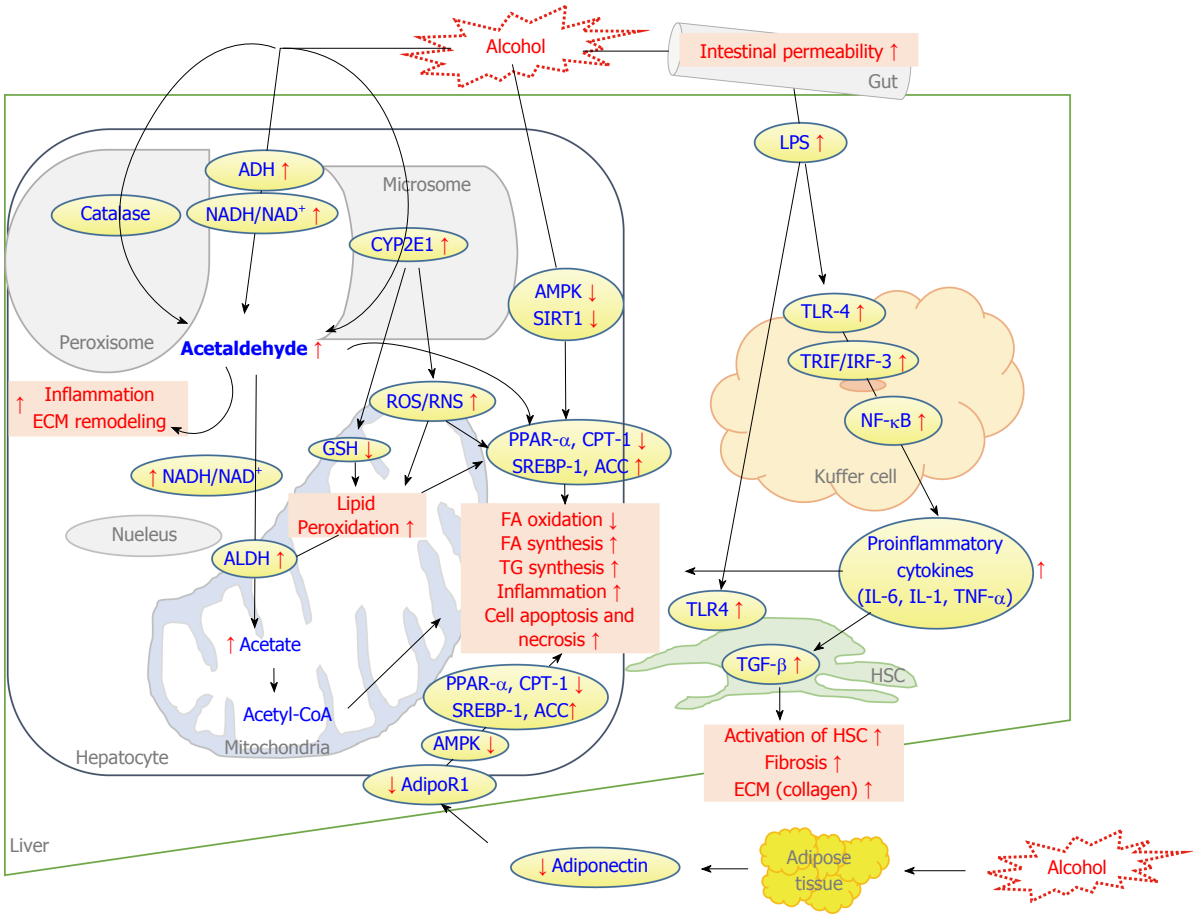

Figure 1 The molecular mechanisms of alcohol-related liver pathogenesis and therapeutic targets. Adapted from references[8,13,159]. When alcohol intake is chronic and heavy, alcohol oxidation occurs *via* cytochrome P450s, resulting in increased levels of CYP2E1, which in turn causes oxidative stress through the generation of ROS which are responsible for lipid peroxidation and alcoholic liver injury. ROS also negatively regulates the activation AMPK and leads to overexpression of SREBP-1, resulting in an increase of *de novo* lipogenesis. GSH also has been reported to its depletion by CYP2E1 followed by the development of oxidative stress. Alcohol consumption negatively affects adiponectin secretion from adipocytes then causes inactive AMPK pathway, leading to elevated *de novo* lipogenesis and inflammatory process in the liver, while simultaneously decreasing fatty acid β-oxidation and contributing to hepatocyte necrosis. The red font indicates the end-point of pathology in the liver. ACC: Acetyl-CoA carboxylase; ADH: Alcohol dehydrogenase; AdipoR1: Adiponectin 1; ALDH: Acetaldehyde dehydrogenase; AMPK: 5' adenosine monophosphate-activated protein kinase; CYP2E1: Cytochrome P450 2E1; CPT-1: Carnitine palmitoyl-transferase-1; ECM: Extracellular matrix; FA: Fatty acid; GSH: Glutathione; HSC: Hepatic stellate cells; IRF-3: Interferon regulatory factor 3; IL-6/IL-1: Interleukin 6 and 1; LPS: Lipopolysaccharide; NADH/NAD+: Nicotinamide adenine dinucleotide hydrogen, nicotinamide adenine dinucleotide; NF-κB: Nuclear factor kappa; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; PPAR-α: Peroxisome proliferator-activated receptor-α; SIRT1: Sirtuin 1; SREBP1: Sterol regulator element binding protein 1; TG: Trigylcerides; TLR-4: Toll-like receptor 4; TNF-α: Tumour necrosis factor-α; TGF-β: Tissue growth factor-β.

abstinence from alcohol[2]. This becomes increasingly vital as the condition progresses. However, this is largely dependent on patient willingness and compliance. Currently, there is no universally accepted therapy to prevent or reverse the progression of ALD. Successful managements include lifestyle modification with the correction of nutritional deficiencies, conventional drugs, liver transplantation, application of complementary medicines, and their combinations[15,16].

Lifestyle intervention

Lifestyle intervention initially entails the abstinence from alcohol. Abstinence limits the development of hepatic steatosis (HS) and prevents further ongoing liver injury, fibrosis and the possibility of HCC. It was reported that abstaining from alcohol improves the prognosis of ALD patients and prevents advancement to hepatic cirrhosis by reducing portal pressure and histological lesions[17,18]. However, long-term alcohol abstinence heavily relies on patient compliance and willingness and therefore may need to be accompanied with psychological therapy or social support. Cognitive behavioral therapy, one of the psychotherapies, reduces the risk of alcoholism and motivates patients to take responsibility and avoid relapse[19]. Pharmacotherapy in combination with psychosocial interventions also encourages patients to sustain abstinence from alcohol. Naltrexone and Acamprosate have been used to manage alcohol abuse in chronic heavy drinkers[20]. Disulfiram has also been approved by the FDA for the management of alcoholism and is broadly utilized despite unclear results from clinical trials[21]. Disulfiram is an acetaldehyde dehydrogenase inhibitor and may cause an increase of serum acetaldehyde by changing

the alcohol metabolism. Disulfiram, therefore, needs to have supervised administration[21]. Furthermore, reducing alcohol consumption, but not completely stopping has also displayed favourable results for the survival of patients with ALD[22]. Other important lifestyle changes can include smoking cessation, adopting a balanced diet and weight control if relevant. Smoking is an independent risk factor for increasing the severity of ALD and its progression to fibrosis and HCC[23,24]. Obesity is another risk factor that can accelerate the progression of ALD as it alone is linked to fatty liver disease and cirrhosis[25].

Nutritional supplements

Malnutrition is another major complication of ALD as poor dietary intake from anorexia, vomiting, mal-digestion, iatrogenic causes, metabolic disturbance, hypermetabolic state, impaired protein synthesis, or mal-absorption can lead to malnutrition and contribute to the disease[26,27]. Nutritional supplementation may address calorie, protein, and nutrient intake to support hepatocyte regeneration[28]. Protein energy malnutrition can be detected with symptoms of muscle wasting, edema and loss of subcutaneous fat. It is recommended that patients with ALD have a daily intake of 1.5 g/kg and 35-40 kcal/kg per day of protein[29]. Supplementation with vitamins such as folate and thiamine should be considered if their deficiencies are detected[16,30]. A discussion concerning micronutrient supplementation is necessary beyond the scope of this article. Nevertheless, an example of micronutrient deficiency related to ALD is seen in zinc deficiency as zinc supplementation has shown to improved ALD by enhancing the activity of ADH and suppressing CYP2E1 in animal models[31,32]. In summary, nutritional status should be balanced in individual cases of malnutrition.

Established conventional therapies for ALD

Although patients living with ALD are mostly treated with strategies to encourage abstinence from alcohol, some patients may need to be accompanied with pharmacological treatment approaches. There are several drugs that have been widely used and reviewed with efficacy, molecular mechanisms (Figure 1), and safety. Table 1 summarizes the effects and mechanism of conventional medicines.

Corticosteroids were one of the first pharmacologic therapies investigated for the treatment of AH and many researchers have extensively studied their use for patients with ALD[33]. The underlying mechanism of action is thought to be the ability of corticosteroids to ameliorate the characteristic inflammatory response by decreasing TNF-α, IL-6, and IL-8[34,35] and also to suppress the formation of acetaldehyde adduct metabolites with inhibition of collagen[36]. Prednisolone, belonging to the corticosteroid family, with a dose of 40 mg/d for 28 d followed by tapering over 2-4 wk is recommended to treat severe alcoholic hepatitis (AH). Another recent published clinical trial randomized 101 acute AH patients to corticosteroids (*e.g.,* prednisone 30 mg/d or methylprednisone 24 mg/d intravenously) and at 30 d, survival was 70% in corticosteroid group when compared to the control (37/53)[37]. Although, corticosteroids have been established as a common treatment for ALD, the data on treatment duration and efficacy of corticosteroids in ALD are conflicting. Long-term usage of corticosteroid seems to enhance the efficacy while others have showed no benefit of corticosteroid therapy after more than 6 mo[38]. Glucocorticoid has been demonstrated to decrease serum bilirubin levels, which is shown to be a clinically useful indicator[39,40]. Using corticosteroids in severe AH, however, carries contraindication including sepsis, hepatitis B, hepatorenal syndrome and gastrointestinal (GI) bleeding[37,41].

Pentoxifylline (PTX), has been thought of as an appropriate substitute to replace corticosteroid treatment in patients with severe AH when there is a contraindication to steroids and is suitable for short-term use[42,43]. The exact mechanism of PTX is not entirely clear. Anti-TNF actions may explain part of its protective effect for ALD. Interesting results have proved that 100 to 1000 μg/mL of PTX inhibited TNF expression by murine peritoneal exudate cells treated with endotoxin 1 μg/mL and PTX treatment significantly reduced serum TNF-α levels in murine *in vivo*[44]. Despite these positive efficacies, treatment with PTX still remains controversial. It has been reported that no significant differences between PTX and corticosteroid treatment groups, or their combination were observed[45]. Meta-analysis also argued that PTX decreased the hepatic-related mortality, but trial sequential analysis did not support this result[46]. Nevertheless, the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases have both recommended that PTX should be considered when AH patients have contraindications to corticosteroid treatment[47].

S-adenosyl-L-methionine (SAM) operates as a methyl donor and contributes in the synthesis of the major cellular antioxidant, glutathione (GSH). In patients with AH and cirrhosis, decreased hepatic SAM, cysteine, and GSH levels caused by abnormality of hepatic gene expression in methionine and GSH metabolism is observed[48]. SAM deficiency arises in early stages of ALD. SAM supplementation can however increase reduced SAM concentrations and reverse liver injury and mitochondrial damage[49]. SAM works to reduce the severity of oxidative stress and hepatic stellate cell (HSC) activation by inhibiting hepatic TGF-α signalling pathway in an ethanol-lipopolysaccharide-induced fibrotic rat model[50]. SAM supplementation has also shown its benefit in 220 patients with chronic hepatitis and cirrhosis in a prospective, double-blind, placebo-controlled trial. Other studies have contradicted these findings, as one demonstrated that there was no significant difference between SAM and placebo in liver

Table 1 The information of effects of current conventional medicines on the treatment of alcoholic liver disease

Medicine/ compound	Type of model	Dosage/duration	Effects	Mechanisms	Ref.
Corticosteroid	RCT in severe AH	Prednisone (30 mg/d, 30 d)	Lower mortality AH group compared to antioxidant treated group		[37]
	RMT and double blind trials in severe AH	Methylprednisolone (32 mg within 7 d) and 28 d treatment and then tapered over 2 wk and discontinued	Decrease short-term mortality of patients with AH		[160]
Pentoxifylline	A double-blind, placebo-controlled trial	400 mg orally 3 times daily for 4 wk	Decrease in the risk of Progressing hepatorenal syndrome; Improve short-term survival in patients with AH		[42]
	A RCT followed by open study in severe AH patients	400 mg/d, orally for 4 wk and then tapered by 5 mg/wk for 7 wk	Better reduced mortality compared to prednisolone in AH		[161]
SAM	Ethanol-LPS-induced fibrotic rats	10 mg/kg bw, ip	Attenuate oxidative stress and HSC activation	Inhibition of TGF-β signaling pathway	[50]
	Double-blind, placebo-controlled trial in 220 patients with AH and cirrhosis	1600 mg/d orally	Decreased serum markers of cholestasis; Improved subjective symptoms such as pruritus, fatigue, and feeling of being unwell		[162]
PPAR-α agonists	C57BL/6J mice with ethanol (27.5% of total calories)	0.1% Wy-14643 for 4 wk	Decreased serum and hepatic fat accumulation	Enhancement of hepatic FA oxidation pathway; Increased PPAR-α mRNA expression by 5-fold	[163]
	C57BL/6J mice with 4 % ethanol (Ethanol feeding for 12 wk)	Wy-14643 (50 mg/kg per day) for last 2 wk	Decreased serum AST and ALT Dropped on score of hepatic steatosis and hepatocyte ballooning	Repress PI3K and COX-2 expression; Enhance adiponectin and HO-1; Increase SIRT1 protein expression	[164]
	C57BL/6J mice with ethanol containing diet (29% of total calories)	Rosiglitazone (3 mg/kg bw/d) for 2 wk	Reduced hepatic triglyceride content; Reduced AST and ALT	Upregulated level of adiponectin and its receptors; Enhance SIRT1-AMPK signaling pathway	[55]
Metformin	C57BL/6 mice TNF knockout mice PAI-1 knockout with ethanol (6 g/kg ig)	200 mg/kg ip for 4 d prior to ethanol administration	Reduced ALT; Prevented hepatic fat accumulation	Prevention of the upregulation of PAI-1	[57]
Metadoxine	HepG2, CFSC-2G and HSC treated with ethanol (50 mM) and acetaldehyde (175 μM)	10μg/mL for 24 h	Reduced peroxidation and oxidized GSH content	Decrease collagen secretion and IL-6, IL-8 and TNF-α secretion	[69]
Carvedilol	Male Wistar rats with ethanol (5%) fed	10 mg/kg per day for last 7 d of total 49 d	Reduced hepatic TG level and the accumulation of fatty droplets within hepatocytes; Inhibited ethanol-induced the thickening of zone 3 vessel walls	Down-regulated FAS and SREBP-1, and up-regulated PPAR-α; Reduced the activation of HSCs with decrease induction of TGF-β1 and collagen	[56]
Propylthiouracil	Male Sprague-Dawley rat with ethanol consumption (36%)	50 mg/kg per day of Wy-14643 for last 2 wk for the last 5 d of 4-6 wk diets with alcohol	Increased hepatic blood flow; Reduces oxidative stress		[71]
Colchicine	RCT in 74 patients with cirrhosis	1 mg/d for 4.4 yr	Reduced serum N-terminal peptide of type Ⅲ procollagen levels		[70]

AH: Alcoholic hepatitis; AMPK: 5′ adenosine monophosphate-activated protein kinase; ALT: Alanine transaminase; AST: Aspartate aminotransferase; bw: Body weight; COX-2: Cyclooxygenase-2, FA: Fatty acid; FAS: Fatty acid synthase; GSH: Glutathione; HepG2: Hepatoma G2 cell-line; HO-1: Heme oxygenase-1; HSC: Hepatic stellate cells; IL-6: Interleukin 6; IL-8: Interleukin-8; LPS: Lipopolysaccharide; mRNA: Messenger ribonucleic acid; P13K: Phosphoinositide 3-kinase; PAI-1: Plasminogen activator inhibiter-1; PPAR-α: Peroxisome proliferator-activated receptor-α; RCT: Randomized controlled trial; RMT: Respiratory muscle training; SIRT1: Sirtuin 1; SREBP1: Sterol regulator element binding protein 1; TG: Triglycerides; TGF-β: Tissue growth factor-β; TNF: Tumour necrosis factor; TNF-α: Tumour necrosis factor-α.

biopsies[51] and no improvement to liver histopathology scores or steatosis, inflammation, fibrosis in ALD patients[52]. Large and high-quality clinical trials are needed to further prove clinical benefits of SAM in ALD.

Peroxisome proliferator-activated receptor-alpha (PPAR-α), a member of the nuclear receptor superfamily and mainly expressed in liver, participates in the regulation of the transcription of genes involved in fatty acid (FA) oxidation, FA transportation, and export of free fatty acid. Alcohol intake inhibits FA oxidation *via* the suppression of PPAR-α in hepatocytes[53]. PPAR-α agonists, are therefore a potential therapy to reverse fat accumulation in AFLD. Many PPAR-α agonists have been assessed as a therapeutic approach to patients with ALD owing their positive efficacies to their anti-inflammatory and hypolipidaemic effects, as well as the induction of FA oxidation. Adiponectin and its receptors, recently, are a new prospective target to manage ALD through PPAR-α, TNF-α and sirtuin 1 (SIRT-1)-related pathways[54]. Thereby, drug discovery of adiponectin agonists may be a potential strategy to manage ALD. PPAR-α plays a pivotal role in the regulation of adiponectin level and rosiglitazone, one of the PPAR-α, agonists has shown to increase the circulating level of adiponectin and enhance hepatic adiponectin receptors (AdipoRs) in ethanol-fed mice[55]. These increments are correlated with the activation of the SIRT-1-AMPK signalling system. Carvedilol, a beta-blocker, has been recently reported to attenuate the development of AH by decreasing hepatic trigylceride (TG) levels and lipid droplets within hepatocytes through down-regulation of fatty acid synthase (FAS) and sterol regulator element binding protein 1 (SREBP-1), and up-regulated PPAR-α[56]. Carvedilol also prevented ethanol-induced thickening of zone 3 vessel walls and reduced HSC activation, decreased induction of TGF-β1 and collagen[56]. Metformin, an antidiabetic drug, has recently been discovered to improve HS by lowering fat accumulation and liver damage in mouse models of acute and chronic alcohol stimulation. This was through the prevention of the up-regulation of plasminogen activator inhibitor-1 and improvement of insulin resistance and liver injury by increasing PPAR-γ and adiponectin levels[57,58].

Although CYP2E1 inhibitors prevent alcohol-induced liver damage, compounds, which have been available in commercial markets, have been too toxic for clinical use. Polyenylphosphatidylcholine (PPC), an innocuous mixture of polyunsaturated lecithins, extracted from soybeans, was recently showed to reduce CYP2E1 activity and also attenuate hepatic oxidative stress and fibrosis[59]. However, one randomized controlled trial (RCT) did not confirm the clear association with the progression of liver fibrosis[60]. Further studies into the effect of PPC on ALD should be encouraged. Clomethiazole, another potential CYP2E1 inhibitor, has also shown to protect the liver from injury in ethanol-fed rats and humans[61,62].

The liver and GI tract shares a reciprocal relationship and therefore establishing healthy gut microbiota has been put forward as a strategy in the treatment of ALD. Lipopolysaccharide (LPS) binds to CD14 in Kupffer cells which reacts with toll like receptor-4 (TLR-4) to activate and release pro-inflammatory cytokines genes[63]. LPS signaling and gut microbiota can be adjusted by probiotics, prebiotics and TLR-4 antagonists to treat ALD[64]. In animal experiments, treating with antibiotics or lactobacillius improved ALD by reducing the gut microflora which suggests that gut-derived endotoxin/LPS may be important in the study of ALD[65]. The modified probiotic, *Escherichia coli* Nissle 1917 was shown to secrete pyrroloquinoline quinone (PQQ), which decreased lipid peroxidation and increased GSH to have an anti-inflammatory, anti-oxidant and anti-hyperlipidaemia effect in acute alcohol exposure in rats[66]. LPS antibody may also benefit injury from LPS induced by exposure to microorganisms in the gut. The outcome of experimental studies has provoked the need to compare the effects of LPS antibodies when combined with corticosteroids for translation into clinical trials for AH[67].

As mentioned, oxidative stress is regarded as a key player in the pathogenesis of ALD with increasing evidence of its importance, such as products of lipid peroxidation being detected in both heavy drinkers and ALD patients[68]. It, therefore, has been anticipated that anti-oxidant therapy would benefit the outcomes for ALD patients. In an animal study, metadoxine had preventative effects of lipid peroxidation and GSH depletion in human hepatocyte (HepG2), exposed with ethanol and acetaldehyde[68]. In HSC, metadoxin also prevents the increase of collagen production and TNF-α secretion[69].

In addition, some drugs have been used for alcoholic liver cirrhosis or fibrosis. Colchicine when administered (1 mg/d) to patients with chronic liver cirrhosis over a three year period displayed anti-fibrotic effects[70]. Propylthiouracil (PTU), typically used as an anti-thyroid medication may also ameliorate liver fibrosis. PTU has been suggested as a therapeutic drug to treat alcohol-induced liver cirrhosis as it increases hepatic blood flow and also reduces oxidative stress in rats[71].

Approach to late stage ALD

Liver transplantation is an effective therapy and is the next viable option for ALD patients with alcoholic cirrhosis who have not responded to abstaining from alcohol. Liver transplantation proposes to be a rescue option for patients with decompensated liver injury and no response from pharmaceutical therapy[72]. Liver transplantation is regarded as a final treatment option for end-stage ALD. However, many reviews have reported that relapse incidences following liver transplantation are common and generally occurs in approximately 10%-52%[73,74] of patients. Moreover, patients who have been treated with liver

transplantation related to ALD display an increased likelihood of *de novo* cancer in other organs, especially, a high rate of cardiovascular complications[75-77]. In addition, the transplantation should be followed after six months of abstinence to avoid histological damage[78,79].

Safety issues with drug treatment

Chronic alcohol uses and subsequent injury to the liver involves various changes to cells and molecules that can compromise the metabolic capabilities of the liver. Therefore, medications that rely mainly on the liver for their metabolism and clearance should be used with caution as there is subsequently less elimination and higher plasma concentrations[80]. Impaired drug metabolism may also relate to changes of the enzyme, CYP2E1[81] which is effected by chronic alcohol use. Additionally, in a cirrhotic liver, drug administration may also be compromised with an increased risk for adverse drug reactions. Furthermore, adverse reactions in the treatment of ALD may often occur due to individual differences such as food intake, genetic differences, age, gender and environmental factors including obesity and diabetes. Although it was found that there was no significantly increased risk of hepatotoxicity in patients with liver disease[80], there still should be considerable attention to dosage when medicating ALD patients to minimise the risk of adverse drug reactions and hepatotoxicity. Caution, for example, should also be taken when using corticosteroids and anti-TNF medications[82] in the hospital setting due to an increased risk of immune-related events including pneumonia, staphylococcus septicaemia, candida septicaemia and GI bleeding[83]. This outcome was seen in a previous study investigating the effects of infliximab and prednisolone which was terminated prematurely due to a significantly higher occurrence of severe infections in the treatment group when compared to placebo and prednisolone[83]. Corticosteroids also increase the risk of coincidental bacterial infections, gastrointestinal bleeding, or renal failure indicating the need for more clinical evidence to confirm this safety issue. Metformin and alcohol combination may produce severe consequences as a side effect of lactic acidosis. This condition can generate when the blood has insufficient oxygen, which is required to transport the glucose throughout the body. Metformin also should be prudent in patients with cirrhosis due to arterial hypoxemia[84]. Other drugs lack evidence in human models beyond preliminary trials due to toxicity or no significant treatment effect. This could perhaps be influenced by the high morbidity rates and short-term mortality associated with end stage ALD, especially acute AH. This highlights the need for complementary and alternative medicine (CAM) where side effects and potential for toxicity are significantly reduced.

Natural medicines as a potential alternative treatment of ALD

CAM encompasses a wide range of approaches with many advantages as it is readily available, has fewer side effects and is a natural way of healing with lasting effects when compared to conventional medicines[85]. With these benefits, natural therapy and herbal medicines have attracted a lot of attention as a potential therapy for the treatment of ALD. It was found that 41% of outpatients with liver disease had utilized some form of CAM[86]. In the US survey, Silymarin and garlic were reported as the most used herbs for liver disease with 12% and 8% respectively. Ginseng (6%), green tea (5%), gingko (5%), echinacea (5%), and St. John's wort (4%) have also been reported. Other than silymarin, the only herb used specifically for liver diseases was licorice root (1%)[87]. Herbal medicines and formulations are commonly grouped according to country. This includes Traditional Chinese Medicine (TCM), Japanese Herbal Medicine, Ayurvedic Medicine (Indian Subcontinent), Traditional African Medicine, Traditional Medicines of the Amazonian Basin in South America, and Arab Traditional Medicine. In general, the markers to display the benefits of herbal products rely on serum/plasma enzymes such as alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Other characteristics, including lipid lowering effects, anti-oxidant, anti- inflammation and anti-fibrotic effects are also used to show the hepatoprotective effects[87]. This review may describe the potential role and effects of natural products in chronic ALD including biological properties and their beneficial effects. A summary list of natural compounds is shown in Table 2.

Green tea polyphenols

Green tea is derived from the leaves and buds of *Camellia sinensis*. Green tea contains rich polyphenolic compounds and has pleiotropic effects including anti-oxidant, hypolipidaemic and anti-inflammatory actions. Green tea polyphenols (GTP) significantly ameliorated ethanol-induced hepatic necrosis. Furthermore, while ethanol significantly increased the accumulation of 4-hydroxynonenal, a product of lipid peroxidation and an index of oxidative stress, green tea extract down regulated this effect. This indicated that GTP protects alcohol-induced liver injury through preventing oxidative stress[88]. The major tea catechins include epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) with EGCG being the major constituent of the catechins with numerous biological functions[89-91]. Our previous study showed total GTP and EGCG enhance glycogen synthesis and inhibit lipogenesis in hepatocytes[92]. EGCG treatment also significantly decreased elevated serum ALT in alcohol-induced rats[88] and normalised the activities of enzymatic anti- oxidants[93]. Another study has reported that EGCG ameliorated plasma endotoxin levels in alcohol-induced female rats with endotoxemia, displaying anti- inflammatory effects through the inhibition of TNF-α, COX-2, and iNOS mRNA expression[94]. EGCG also protected against liver impairment through the enhancement of FA oxidation

Table 2 The information of effects of current natural medicines on the treatment of alcoholic liver disease

Medicine/ compound	Type of model	Dosage/duration	Effects	Mechanisms	Ref.
Vitamin E and C	Malnourished rats with ethanol	Vitamin E (15 mg/kg) Vitamin C (10 mg/kg); single and combined treatments	Decreased ethanol induced hepatic glutathione peroxidase activity and hepatic fibrosis; Attenuated the development of hepatomegaly and hepatic necroinflammation		[165]
Epigallocatechin-3-gallate (EGCG)	Male albino Wistar rats with Ethanol (6 g/kg per day) for 60 d	EGCG (100 mg/kg per day) for the last of 30 d of ethanol administration	Normalization of activities of enzymatic antioxidants; Reduction of lipid peroxidation		[93]
	Female Sprague-Dawley rats with ethanol (56%)	EGCG (100 mg/bw)	Partly blocked the gut leakiness; Reduced endotoxemia and lipid peroxidation	Blunted the elevated expressions of CD14, TNF-α, COX-2 and iNOS	[94]
	Male Wistar rats with ethanol for 5 wk	EGCG contained diet (3 g/L) for 2 wk and then ethanol-EGCG diet for 5 wk	Reduced serum AST and ALT	Enhancement of FA oxidation through increasing of CTP-1 and p-ACC expression	[95]
Silymarin (*Silybum marianum*)	RCT, double-blind, 170 patients with cirrhosis	140 mg/d for 3 times orally	Reduced the rate of mortality No side effect		[33]
	Alcohol induced baboons (50% of calories)	0.84 mg/calorie for 36 mo	Improve histologic stage of fibrosis	Decrease collagen I and (I) procollagen	[100]
	Alcohol and high fat induced rats	100 mg/kg per day, 150 mg/kg per day, 200 mg/kg per day for 6 wk	200 mg/kg per day decrease serum ALT and AST and hepatic fat contents	Attenuated NF-κB p65, ICAM-1 and IL-6 were found in silymarin groups (150 mg/kg, 200 mg)	[101]
Betaine	Ethanol diet Wistar rats	1% (w/v) in diet	Decrease hepatic ballooning and fat contents	Attenuate NOS production; Decrease CYP2E1 protein and activity	[106]
	High fat containing ethanol diet rats 6 g/kg for 8 more wk	200 and 400 mg/kg per day for 4 wk	Decrease ALT and AST	Inhibition of TLR-4 expression; Decrease serum endotoxin, TNF-α, IFN-γ and IL-18	[107]
Glycyrrhizin (*Glycyrrhiza glabra*)	Ethanol-CCl_4 induced male SD rats	Intraperitoneal injections of potenlin (acquired from Hai Ning Pharmaceutical Co., Zhe Jiang, China)	Decreasing serum ALT levels	Normalized NF-κB binding activity	[114]
Ginsenosides	Ethanol-feeding mice Ethanol feeding hepatocytes (AML12 cell lines)	Red ginseng extract containing abundant ginsenosides (Rb1, Rb2, and Rd) (250 mg/kg or 500 mg/kg) for 4 wk in mice	Improves chronic alcohol-induced histopathological changes; Decreases in hepatic triglyceride content	Inhibition of lipogenesis pathway; Attenuated EtOH-induced cytochrome; P450 2E1, 4-hydroxynonenal, and nitrotyrosine levels; Activation of AMPK-SIRT1	[166]
	Alcohol consumption with high fat diet mice	Red ginseng (200 mg/kg per day) for last 2 wk	Lower ALT levels and no different AST	Reduced level of TNF-α and IL-1 and increase IL-10	[124]
Fenugreek seed polyphenol	EtOH (30 mM) induced Chang liver cells	20, 40, 60 mg/mL	Increased GSH/GSSG ratio Reduced EtOH-induced LDH leakage	Inhibition of NF-κB	[129]
	Ethanol induced rats (6 g/kg per day) for 30 d	200 mg/kg per day for 30 d	Improved lipid profile and reduced collagen content, crosslinking, aldehyde content and peroxidation		[132]
Curcumin	Alcohol (100 mM) induced rat primary hepatocytes	0-50 μmol/L	Ameliorated MDA and AST	Improved GSH and heme oxygenase-1 (HO-1) induction	[133]
	Alcohol-induced female Sprague-Dawley rats	400 mg/kg bw	Improved liver pathology, decreasing elevation of hepatic MDA	Suppressing of NF-κB activation	[134]
LIV-52	Ethanol-induced HepG2 cells (100 and 200 mM)	1% or 2% Liv.52 for 24 h		Normalized suppressed PPAR-α and induced TNF-α	[148]

ACC: Acetyl-CoA carboxylase; AML12: Alpha mouse liver 12; AMPK: 5′ adenosine monophosphate-activated protein kinase; ALT: Alanine transaminase; AST: Aspartate aminotransferase; bw: Body weight; CCL4: Chemokine ligand 4; CD14: Cluster of differentiation 14 gene; COX-2: Cyclooxygenase-2; CPT-1: Carnitine palmitoyl-transferase 1; CYP2E1: Cytochrome P450 2E1; EGCG: Epigallocatechin-3-gallate; etOH: Ethanol; FA: Fatty acid; GSH: Glutathione; GSSG: Glutathione disulfide; HepG2: Hepatoma G2 cell-line; HO-1: Heme oxygenase-1; ICAM-1: Intercellular adhesion molecule-1; IFN-γ: Interferon-γ; IL-1: Interleukin 1; IL-6: Interleukin 6; IL-10: Interleukin-10; IL-18: Interleukin-18; iNOS: Inducible nitric oxide synthase; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa; NOS: Inducible nitric oxide synthase; RCT: Randomized controlled trial; SIRT1: Sirtuin-1; TLR-4: Toll-like receptor-4; TNF-α: Tumour necrosis factor-α.

with increased carnitine palmitoyl transferase 1 (CPT-1) levels[95]. Despite much evidence supporting the effects of EGCG on ALD, clinical studies are still limited and large-scale human studies may be needed to confirm this effect. GTP, catechins may also increase HO-1 activity to reduce oxidative stress in human liver tissue (hHeps). Administering polyphenols prior to ethanol exposure reduced the diminishing of GSH to display a protective effect on liver cells by regulating the immune system and pro-inflammatory mediators such as TGF-β1[96].

Herbal medicines

Silymarin (Milk thistle), which is one of the active compounds extracted from *Silybum marianum* (Asteraceae) is the most common type of CAM therapy for treatment of liver diseases[85]. Silymarin is a complex mixture of polyphenolic molecules including 7 flavonolignans (silybinA, B, isosilybin A, B, silychristin, isosilychristin, silydianin) and 1 flavonoid (taxifolin)[97]. Silibinin, which is a semi-purified fraction of silymarin, has been shown to be a radical scavenger[98] and a RCT with silymarin also improved survival from patients with alcoholic and non-alcoholic cirrhosis without any side effect[33]. Another RCT was also conducted with the prospective effect of silymarin (140 mg/d) in AH[99]. Mechanisms by which silymarin extracts ameliorate liver disease include anti- inflammatory, anti-oxidative, antifibrotic, and immune-modulating activities[100]. Silymarin has retarded the development of alcohol-induced hepatic fibrosis in baboons by preventing collagen type Ⅰ and decreasing histological progression to fibrosis[100]. Silymarin also reduces the elevated serum AST and ALT levels, and decreases hepatic lipid contents in alcohol-induced fatty liver in rats. These effects may be through down-regulating the expression of NF-κB p65, ICAM-1 and IL-6 in liver tissue[101]. However, Cochrane Database reviews have been unsuccessful in demonstrating the beneficial effect of silymarin on ALD. Further studies are needed to provide the appropriate data concerning efficacy.

Betaine is another natural dietary compound, synthesized *in vivo* from choline and converts homocysteine to methionine[102]. Betaine (trimethylglycine), a pivotal nutrient for humans, can be acquired from a wide range of foods and nutritional supplements[103]. It attenuates AH by restoring phosphatidylcholine generation *via* the phosphatidylethanolamine methyltransferase pathway[104]. Betaine also increases SAM, normalizing hepatocellular SAM:S-adenosyl-*L*-homocysteine ratio[105], resulting in the attenuation of fatty liver[103]. The study of mitochondrial dysfunction in the progression of ALD has also been enforced by many research areas. Betaine gives a beneficial effect against loss of oxidative phosphorylation system proteins by alcohol, by preventing NOS_2 induction and NO generation in male Wister rats with exposed to ethanol[106]. The mechanism of betaine in liver is also involved in the suppression of endotoxin/TLR-4 signaling pathways. Betaine treatment decreased the levels of serum ALT, AST, endotoxin, TNF-α, IFN-γ, IL-18, and TLR-4, and improved the degree of HS and inflammation in liver tissues in rats with alcohol-induced liver injury[107].

Glycyrrhiza glabra (Leguminosae), commonly named licorice root, is a perennial herb cultivated in temperate and subtropical regions of the world as well as central and South-Western Asia[108]. Glycyrrhizin, as a main constituent in aqueous extract, is a conjugate of two molecules of glucuronic acid and one of 18β-glycyrrhetic acid[109]. Glycyrrhizin is hydrolysed by intestinal bacterial into 18β-glycyrrhetic acid, which is reabsorbed into the bloodstream. Glycyrrhizin suppressed serum AST and ALT levels and histologically inhibited the spread of deteriorating areas in hepatocytes in an animal model of concanavalin A-induced liver injury[110]. Moreover, the effects of Glycyrrhizin has extended to the attenuation of inflammation response by regulation of NF-κB and MAPK pathway[111] and inhibition of TNF-α, ROS, and proinflammatory interleukins such as IL-6 and IL-1β[112,113]. Another beneficial effect of glycyrrhizin, from Potenlini compound, was shown in ethanol-CCl_4 induced liver cirrhosis rats by decreasing serum ALT levels and normalising NF-κB binding activity[114,115]. In clinical trials, glycyrrhizin is mostly used to treat hepatitis C and interferon treatment[16]. One clinical trial showed to lower level of ALT with 200 mg of glycyrrhizin for 5 d per week for approximately 10 years in hepatitis C patients[116]. Glycyrrhizin has potential effects to cure various chronic liver diseases but large-scale, rigorously designed clinical trials for ALD is warranted.

Ginseng, an ancient medicinal herb, has been popular as a tonic for the treatment of various diseases including diabetes and hepatic diseases[117]. Ginsenosides, the main active constituent groups of all ginseng species, has been used as a supplementary medicine and is mostly responsible for the beneficial pharmacological effects in metabolic disorders and various cancer *via* its ability to boost immunity and its anti- inflammation function[118,119]. Ginsenoside Rb1, as the most abundant ginsenoside in *P. ginseng* has improved hepatic fibrosis induced by CCl_4 by down regulation of hepatic prostaglandin E2 and TIMP-1[120]. Ginsenoisde Rg1 showed to inhibit TNF-α-mediated NF-κB transcriptional activity in HepG2 cells with IC50 of 28.14 μmol/L and gene expression of iNOS and COX-2 inducible inflammatory enzymes[121]. It was also reported that ginsenoside Rc (40 mg/kg) attenuates AFLD in alcoholic-fed ICR mice through the regulation of AMPK and MAPK pathways[122]. Red ginsengs, which contain an abundance of ginsenosides, showed to improve chronic alcohol-induced histopathological changes and hepatic TG content through inhibition of the lipogenesis pathway and AMPK-SIRT1 activation in alcohol-fed mice[123]. Red ginseng also reduced levels of TNF-α and IL-1β which were increased by alcohol consumption with a high fat diet in mice[124]. Recently,

compound K which is a metabolite form of ginsenoside Rb1 and Rc has been identified as an inducer to increase AMPK expression, resulting in reduction of lipid droplets and hepatic TG accumulation in high FA exposed human hepatocytes (Huh7). This compound had better efficacy to treat HS compared to metformin[125]. With the hepatoprotective effects of many types of ginsenosides, these herbal components should be potential targets to improve liver damage caused by alcohol abuse.

Fenugreek (*Trigonella foenum graecum*) seeds have been reported to have beneficial effects on enhancing the antioxidant apparatus[126]. Fenugreek seeds also have been used in remedies for diabetes and high cholesterol in various traditional medicines and proven experimentally in diabetic humans[127]. The polyphenolic extract of the seeds is the main constituent beneficial for the treatment of ALD according to many articles. In general, fenugreek seed polyphenols contain five different polyphenolic flavonoids, vitexin, tricin, naringenin, quercetin, and tricin-7-O-b-D-glucopyranoside[128]. These polyphenols prevent the alcohol-induced toxic effect in human Chang liver cells through improvement of GSH/GSSG ratio[129]. Interestingly, quercetin ameliorated ethanol-stimulated mitochondrial dysfunction by induced permeability transition though suppressing GSH depletion. This indicates a promising preventive strategy for ALD[130,131]. Fenugreek seed polyphenols also have demonstrated improvement to lipid profiles and collagen content in alcohol-fed rats[132]. Compared to other herbal medicines, however, the clinical studies of fenugreek seed polyphenols are lacking evidence in metabolic disorders, especially in relation to ALD, suggesting the need for accurate standardization of polyphenols and their individual efficacy for the treatment of ALD.

Curcumin, commonly called turmeric yellow and compound of *curcuma longa*, is a low-molecular weight polyphenol derived agent. It is commonly used in Ayurvedic medicine and has various pharmacological effects including anti-oxidative, anti- inflammatory, and hepatoprotective activities[105]. This historical use of curcumin has prompted investigation into the hepatoprotective effects against liver damage induced by alcohol abuse and the molecular mechanisms, however the research in this area is still lacking. Curcumin has ameliorated malondialdehyde (MDA) and AST and improved GSH and heme oxygenase-1 (HO-1) induction in alcohol (100 mM) induced rat primary hepatocytes[133]. Curcumin (400 mg/kg bw) has also been examined in alcohol-induced female Sprague-Dawley rats to show improvement by reducing the elevation of hepatic MDA, and the suppressing of NF-κB activation[134]. Another similar result confirmed the inhibition of the expression of NF-κB-dependent genes by the supplementation of curcumin (75 mg/kg per day) for 4 wk in rats[135]. Recently, low dosage of curcumin prevented HS compared with the alcohol control group with inhibition of dehydrogenase, ALDH2 as well as CYP2E1 activation[136]. Curcumin also proved its anti-oxidant effect against ethanol-exposed mice by decreasing ROS generation[137]. Despite the beneficial effects of curcumin in ALD, it is unfavourable to apply as a medical strategy due to its decreased bioavailability and rapid metabolism with systemic elimination in animals and humans, suggesting an investigation of more sophisticated pharmacokinetic improvements of curcumin such as curcumin phospholipid complex or curcumin nanoparticles[105,138].

Herbal formulas to treat ALD

Historically, some herbal blends have been commonly used for hepatic disorders around the world. Clinical trials to evaluate their efficacy are, however, challenging to demonstrate due to heterogeneous formulations and dosages. Moreover, these blends have not been regulated by the Food and Drug Administration. In this review, a few blends are evaluated in ALD. Herbal Medicine861 (HM 861) consists of 10 herbs, based on TCM including *Salvia miltiorrhiza*, *Astragalus membranaceus* and *Spatholobus suberectus*[139]. HM861 has shown the inhibition of HSC proliferation and induction of HSC apoptosis in patients with chronic hepatitis[140].

Moreover, the compound also suppressed tissue inhibitor metalloprotease 1 (TIMP-1) mRNA expression in HSC-T6 cells[141]. In clinical trials, this herbal blend was tested for anti-fibrotic activity encompassing 107 patients with hepatitis B resulting in the drop of ALT levels to the normal range in 73% of patients[142]. In a clinical trial, HM861 showed to improved fibrosis and early cirrhosis in 52 patients with HBV-related fibrotic patients with the hepatic inflammatory scores also decreased[143]. More Cochrane reviews, however, are needed to confirm the efficacy of the blend in the treatment of ALD. TJ-9, also referred to as Shosaiko-to in Japan or xiao-chai-hu- tang in China, containing 7 herbal constituents including: bupleurum root, pinellia tuber, scutellaria toot, jujube fruit, ginger rhizome, ginseng root, and glycyrrhiza root. The formula has been traditionally used for the treatment of liver diseases. Usually, the preparation contains 7.5 g of TJ-9 with the active components, baicalin and baicalein, responsible for the anti-oxidant activity[144]. Although the exact of mechanism of TJ-9 is unknown, one anticipated mechanism may be exposed in the observation of stellate cells which has shown an inhibition of α-smooth muscle actin, type Ⅰ collagen production, and cell spreading, indicating the suppression of HSC activation[144]. TJ-9 has been associated with several cases of interstitial pneumonitis, especially when used with interferon for the treatment of chronic hepatitis[145]. Liv-52, which is an Indian Ayurvedic medicine that has been used for the treatment of liver diseases, is a herbal preparation including *Capparis spinosa* (Himsara), *Cichorium intybus* (Kasani), *Mandur bhasma, Solanum nigrum* (Kakamachi), *Terminalia arjuna* (Arjuna),

Cassia occidentalis (Kasamarda), *Achillea millefolium* (Biranjasipha), *and Tamarix gallica* (Jhavaka)[146]. It was originally used to treat ALD, but a recent RCT from Europe demonstrated a detrimental effect on advanced alcohol-induced cirrhosis[147]. Contrastingly, an *in vitro* study, reported that Liv.52 improved PPAR-γ suppression and TNF-α expression in HepG2 cells[148]. Another clinical study also showed the efficacy of LIV.52 in 26 cirrhotic patients for 6 mo with normalized serum ALT and AST levels compared to the placebo group[149]. Although there are many positive effects of LIV.52 for the improvement of ALD, underlying molecular mechanisms are still necessary to support its potential function. In summary, many compounds are available on the open market to treat ALD, but their efficacy data is still not supported by clinical trials. Comprehension of herbal interaction with pharmacological medicines is also necessary to improve the treatment of ALD.

The combination therapies of drugs and natural agents

More readily available on the market and popular than ever, CAM and herbal therapy are increasingly attractive treatments for chronic liver diseases with many advantages, such as low side effect and toxicity. Augmentation of herb-drug interaction has consequently followed stipulating dialogue between pharmaceutical experts and practician. Although there is strong evidence, supporting the use of herbal medicine for the treatment of ALD, combination therapy should be speculated for any safety issues. Magnesium isoglycyrrhizinate injection is one example of a drug containing glycyrrhizin and possesses effective and safe treatment for chronic liver diseases[150] and down regulates the progress of pulmonary fibrosis[151]. Glycyrrhizin also has been combined with matrine to improve CCl_4-induced liver fibrosis through lowering levels of collagen and less HSC proliferation[152], suggesting the positive effect of their combination to protect against liver fibrosis. Recently, a combination of silymarin and SAM was evaluated in ALD markets with much promise[153]. There have been some cases of adverse events and hepatotoxicity caused by herbal medicines[154]. *Xiao Chaihu Tang*, alone or in combination with interferon, may induce acute interstitial pneumonia in chronic hepatitis patients[155]. Despite the emerging of CAM on the markets, documented herb-drug interactions are still sparse, especially in relation to ALD with a reliance only on individual case reports. Although some combinations of CAMs and conventional drugs contribute many beneficial efficacies, some herb-drug interaction also may be associated with toxicity in certain situations such as the combination of silymarin with indinavir therapy in AIDS patients[156].

FUTURE OUTLOOK AND PERSPECTIVE

It is undeniable that chronic alcohol abuse leads to deleterious implications for the liver regardless of age, gender or other factors. The more advanced the stage of alcoholic liver disease, the more morbid and life-threatening the complications become. Various therapies should, therefore, aim to reverse these complications but are often only palliative. As an initial therapy of ALD, abstinence from alcohol accompanied with basic lifestyle modifications when appropriate. When the injury becomes decompensated, pharmacological therapy should be accompanied to reverse the more severe stages of ALD. The present studies are still inconclusive with humble methodological quality and design, high heterogeneous patient populations, and poorly defined point of progression of ALD. These flaws may partly explain the conflicting reports in the literature to support the effectiveness of treatment for ALD for many conventional medications, such as Corticosteroids, PTX, and SAM. Mechanistic studies are still insufficient to prove their pharmaceutical potency and future studies will be thirsty to investigate the molecular mechanisms related in the complex relationships between ethanol metabolism, oxidative stress, immune response, HSC activation and extracellular matrix remodelling. Larger RCTs with a longer follow-up period are required for further evaluation.

Increasing attention has been paid to herbal medicines as a newly emerging treatment strategy for ALD. In this review, we summarized functionalities of CAM for the prevention and treatment of ALD. Most single herbs and formulae have resulted in improvement of ALD-related conditions with multiple and diverse mechanisms of actions in spite of their mild side effects. To date, only a few active compounds from herbal extracts have been identified as candidates to treat ALD and alcohol induced liver injury. Although many herbal constituents have shown promising potential for the treatment of ALD with multi-targets, the underlying molecular mechanisms, especially that of single herbal compounds, have not been completely elucidated. Moreover, clinical trials to adjust concurrent systematic review are still required in the area of ALD research. More profound studies underlying the prospective effect of herbal medicines should be further investigated. In addition, some unique therapies are now increasing to develop the treatment of ALD. Epigenetic regulation underlying alcohol metabolism, such as histone modification may be a new trend to advance effective treatment strategies for ALD[157]. Acetate may affect histone modification by up-regulating acetyl-CoA and enhance the inflammation in ethanol-exposed macrophages by interfering histone deacetylase activity[158]. With many natural products potentially possessing this ability of epigenetic modification, it would be a particularly beneficial breakthrough for ALD patients.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

Multipotent mesenchymal stromal cells: A promising strategy to manage alcoholic liver disease

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Abstract

Chronic alcohol consumption is a major cause of liver disease. The term alcoholic liver disease (ALD) refers to a spectrum of mild to severe disorders including steatosis, steatohepatitis, cirrhosis, and hepatocellular carcinoma. With limited therapeutic options, stem cell therapy offers significant potential for these patients. In this article, we review the pathophysiologic features of ALD and the therapeutic mechanisms of multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs), based on their potential to differentiate into hepatocytes, their immunomodulatory properties, their potential to promote residual hepatocyte regeneration, and their capacity to inhibit hepatic stellate cells. The perfect match between ALD pathogenesis and MSC therapeutic mechanisms, together with encouraging, available preclinical data, allow us to support the notion that MSC transplantation is a promising therapeutic strategy to manage ALD onset and progression.

Key words: Alcoholic liver disease; Cellular therapy; Alcoholic steatohepatitis; Hepatic function recovery; Mesenchymal stem cells

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Core tip: Chronic alcohol consumption is a major cause of liver disease. Stem cells, in particular multipotent mesenchymal stromal cells (MSCs), have been envisioned as a promising tool for the development of therapeutic strategies to treat alcoholic liver diseases (ALD). The advantages of MSC include the regulation of exacerbated inflammatory process, their differentiation into hepatocytes, the production of trophic factors that prevent the apoptosis of parenchymal cells, and the induction of the proliferation of endogenous progenitors. Here, we revise the pathophysiology of ALD to identify therapeutic targets for MSCs. Also, we discuss the rationale to propose an MSC-based therapy to treat ALD.

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ALCOHOLIC LIVER DISEASE

Chronic alcohol consumption is a major cause of liver disease[1-3]. Moreover, alcohol consumption negatively impacts the natural history of other types of chronic liver diseases such as nonalcoholic steatohepatitis, and hepatitis B and C, favoring fibrosis progression[3-5]. Alcoholic liver disease (ALD) comprises a broad spectrum of disorders, encompassing simple steatosis, steatohepatitis, and cirrhosis. The pathomechanism associated with ALD involves complex interactions between the deleterious effects of alcohol and its toxic metabolites on various cell types in the liver, the induction of reactive oxygen species (ROS), and the up-regulation of the proinflammatory cascade[1,3].

Alcoholic steatosis, the earliest manifestation of ALD, is present in more than 90% of heavy drinkers, and is pathologically characterized by microvesicular and macrovesicular fat accumulation within hepatocytes, minimal inflammatory reaction, and no hepatic fibrosis[1]. This stage is asymptomatic and reversible with alcohol abstinence[6]. Alcohol consumption increases the ratio of NADH/NAD^+ in hepatocytes, which disrupts mitochondrial β-oxidation of fatty acids, leading to steatosis development[7]. Alcohol consumption also increases fatty acid triglycerides synthesis through the upregulation of the sterol regulatory element binding protein 1c[8] and the downregulation of the peroxisome proliferator-activated receptor-α[9]. ALD progression is characterized by steatosis, a superimposed inflammatory infiltrate of predominantly polymorphonuclear leukocytes and hepatocellular damage. When the inflammation and hepatocellular injury are severe, the condition is termed steatohepatitis and is associated with a high mortality rate[10,11].

The pathogenesis of alcoholic steatohepatitis is complex and multifactorial. In the liver, alcohol is metabolized primarily into acetaldehyde by the enzymes alcohol dehydrogenase in the cytosol, cytochrome P450 in microsomes, and catalase in peroxisomes[12]. Acetaldehyde is highly toxic to hepatocytes because it binds to proteins and DNA, forming adducts that promote glutathione depletion, lipid peroxidation, and mitochondrial damage[13,14]. Additionally, these adducts act as antigens that activate the adaptive immune response, leading to lymphocyte recruitment to the liver[15]. Acetate resulting from acetaldehyde breakdown is rapidly released from the liver into circulation and then metabolized into CO_2 *via* the tricarboxylic acid cycle in skeletal muscle, brain, and heart. Although acetate has no direct hepatotoxicity, it is believed that it can regulate the inflammatory response in patients with alcoholic steatohepatitis through the upregulation of proinflammatory cytokines released by macrophages[16].

Alcohol abuse also results in changes in colonic microbiota and increased gut permeability, leading to translocation of bacterial products, such as lipopolysaccharide, into the portal circulation[17]. In Kupffer cells, lipopolysaccharide activates the MyD88-independent signaling pathway through toll-like receptor 4, resulting in the production of oxidative stress and proinflammatory cytokines such as tumor necrosis factor (TNF)-α, contributing to hepatocellular damage[18,19].

Histologic features of alcoholic steatohepatitis include inflammation and necrosis, which are more prominent in the centrilobular region of the hepatic acinus, while hepatocytes are classically ballooned, leading to compression of the sinusoid and portal hypertension[20,21]. Alcoholic cirrhosis is the end stage of ALD and is characterized by distortion of the hepatic architecture, septum formations, rings of scars that surround hepatocyte nodules, the formation of regenerative nodule, and the loss of liver function[22].

Extracellular matrix (ECM), particularly collagen type Ⅰ, is mainly produced by activated hepatic stellate cells (HSCs), located in the space of Disse between the hepatocytes and sinusoids. HSCs can be activated by neutrophils, damaged hepatocytes, and activated Kupffer cells through various profibrogenic mediators, including transforming growth factor (TGF)-β, TNF-α, and ROS[3,23]. Additionally, ROS downregulate the action of metalloproteinases and upregulate tissue inhibitor of metalloproteinase-1, resulting in greater collagen accumulation[24].

Along with other liver diseases, patients with cirrhosis are at risk for hepatic decompensation (ascites, variceal bleeding, and encephalopathy) and the development of hepatocellular carcinoma[25,26]. Although the most important risk factor for ALD is the absolute amount of alcohol intake, only approximately 35% of heavy drinkers develop advanced ALD, indicating that other factors are involved in host susceptibility to the disease. These factors include sex, obesity, drinking pattern, dietary factors, non-sex-linked genetic factors, and cigarette smoking[27-30].

CURRENT ALD TREATMENT

Despite the profound economic and health impacts of ALD, little progress has been made in the management of patients with this condition, and medical treatment has not changed significantly in the last 45 years[10,31,32]. Although nutritional and supportive management are important, alcohol abstinence is the mainstay therapy for patients with all stages of

ALD[33,34]. However, the benefits of alcohol abstinence may not be sufficient for patients with decompensated ALD, such as cirrhosis or severe alcoholic hepatitis[35,36].

Corticosteroids were one of the first pharmacologic therapies investigated for the treatment of alcoholic hepatitis. Despite the widespread awareness and use of this therapy, controversy still exists regarding its true efficacy[37]. Taking into account the participation of TNF-α in ALD pathogenesis, TNF-α antagonists have also been studied for this condition. Although the initial studies were promising, larger clinical trials demonstrated an increased risk of infection and mortality with these agents[38]. In addition, pharmacologic therapy with medications such as disulfiram, baclofen, colchicine, vitamin E, and naltrexone have been considered, although their efficacies are limited[3,39,40].

The most effective therapy for advanced cirrhosis is liver transplant, however, the scarcity of donors, surgical complications, immunologic suppression and rejection, and high medical cost limit its availability and clinical utility[41]. Moreover, liver transplantation is not an option for most patients, and, until now, no other treatment has demonstrated superiority over steroids. Therefore, alternative therapies are needed. To this end, alternative approaches that circumvent the use of the whole organ, such as transplantation of cells of diverse origins, have been proposed in recent years[42].

CELLULAR THERAPY FOR LIVER REGENERATION

It is well known that the liver has a high regenerative capacity. Under normal conditions, recovery of liver mass occurs mainly *via* proliferation of remaining adult hepatocytes. On the other hand, under pathologic conditions in which the proliferation of hepatocytes is inhibited, liver progenitor cells (oval cells) proliferate and differentiate into hepatocytes or biliary epithelial cells[43]. Chronic ethanol exposure and sustained inflammation have been shown to inhibit DNA synthesis in the damaged liver[44,45]. This impaired hepatocyte proliferation is the consequence of oxidative damage by the ROS produced from alcohol metabolism[46]. Moreover, ethanol could inhibit early differentiation of hepatic progenitor cells into functional mature hepatocytes[47].

Cell therapy for the treatment of hepatic fibrosis has been evaluated in different animal models, and some findings have been very encouraging. The transplantation of mature hepatocytes into human patients has provided insights into the way in which human liver disease could be treated by cellular therapies[48]. However, the high number of cells needed for the transplantation, the availability of fresh cells or the quality of cryopreserved ones, and the necessity of immunosuppression to avoid the rejection of transplanted cells are the main limitations of adult hepatocyte transplantation[49,50]. Immunosuppression is a particularly important point, as the hepatic failure itself increases the risk of developing septic complications, which are worsened by the use of immunosuppressive drugs.

Numerous studies have focused on investigating the ability of a variety of stem cells that can be readily isolated using noninvasive procedures to give rise to hepatocytes both *in vitro* and *in vivo*[51]. Considering that some of these stem cell populations are present in adults, it would be possible to produce personalized immunologically matched hepatocytes[52]. Moreover, adult stem cells have the ability to reduce the hepatic proinflammatory microenvironment, inhibit the activation or induce apoptosis of HSCs, and promote the regeneration of residual hepatocytes[53,54].

MESENCHYMAL STEM CELLS AS A TOOL FOR THE INDUCTION OF TISSUE REGENERATION

The aim of regenerative medicine is to develop therapeutic strategies for the management of severe injuries or chronic diseases in patients whose endogenous regenerative mechanisms have failed to restore the impaired functions. Over the past years, stem cells have been envisioned as the best tool for this purpose. Stem cell-based intervention is known to act through multiple mechanisms, which is a clear advantage when facing diseases with a complex pathophysiology, such as ALD.

In general terms, adult stem cells are found in all nonembryonic tissues, where they contribute to both maintenance of cellular homeostasis and regeneration of damaged organs. These cells are multipotent and can be isolated from a fetus, newborn, child, or adult, and due to their limited self-renewal potential, they are not teratogenic. Some of them also have plasticity, *i.e.*, they can differentiate into cells from lineages different from their origin[55].

As adult stem cells pose fewer bioethical and technical concerns than embryonic stem cells, the first candidate for a stem cell-based strategy to treat liver regeneration was bone marrow-derived stem cells[53,56-58]. Bone marrow harbors at least two distinct adult stem cell populations: the hematopoietic stem cells that give rise to blood and endothelial cells[59] and the multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs), that provide support to hematopoietic stem cells and drive the process of hematopoiesis[60]. In addition to bone marrow, MSCs have now been isolated from numerous tissues, including liver, lung, umbilical cord, skeletal muscle, dental pulp, spleen, and adipose tissue[61-63]. Thus, it has been postulated that MSCs play a critical role in organ homeostasis by providing supportive factors to the surrounding tissue.

Table 1 Proposed cellular and molecular mechanisms that could contribute to hepatic protection by mesenchymal stem cells in alcoholic liver disease

MSCs in liver inflammation
Inhibit the proliferation of CD8 cytotoxic T lymphocytes and increase the relative rate of CD4 Th2 lymphocytes[97,100]
Inhibit the maturation of monocytes into dendritic cells[94]
Inhibit the secretion of TNF-α, INF-γ, and IL-12 by dendritic cells and increase their secretion of IL-10, reducing the proinflammatory potential[95]
Suppress the proliferation, cytolytic activity, and cytokine secretions of the NK cells[96]
Express indoleamine 2,3-dioxygenase upon INF-γ stimulation, leading to tryptophan depletion and the inhibition of T-cell proliferation[98]
MSCs in liver fibrosis
Reduce the proliferation of HSCs and the synthesis of collagen type Ⅰ through the secretion of TNF-α[125]
Induce HSCs apoptosis[124]
Express matrix metalloproteinase-9, which degrades the extracellular matrix[128,129]
MSCs in liver regeneration
Secrete trophic factors (HGF, EGF, and IGF-1) that promote hepatocyte proliferation and function during liver regeneration[68,128,130]

EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; HSC: Hepatic stellate cell; IGF-1: Insulin-like growth factor-1; IL: Interleukin; INF-γ: Interferon-γ; MSC: Mesenchymal stem cell; TNF-α: Tumor necrosis factor-α.

One of the main technical difficulties associated with the therapeutic use of MSCs is the lack of a specific antigen for their identification. Therefore, in 2006, the International Society for Cellular Therapy proposed minimal criteria to define human MSCs (hMSCs): (1) must be plastic-adherent when maintained under standard culture conditions; (2) must express CD105, CD73, and CD90, and lack the expression of CD45, CD34, CD14, CD11b, CD19, and class Ⅱ human leukocyte antigen surface molecules; and (3) must differentiate into osteoblast, adipocytes, and chondroblasts under *in vitro* differentiation conditions[64,65].

Despite the scarcity of MSCs (< 0.01% of the mononuclear cells present in the bone marrow), they can be considered as ideal candidates for cell therapy because: (1) they can be obtained from donors without major complications; (2) they can be easily expanded *ex vivo*; (3) when MSCs are systemically administered, they can selectively migrate to and engraft damaged tissue. The migration of MSCs is facilitated by the release of several molecules from the damaged tissues that interact with different receptors expressed by the MSCs[66,67]; (4) it has been suggested that MSCs cross the germ line barrier and generate cells from the endodermal and ectodermal lineages[55]; (5) MSCs secrete a broad range of bio-active growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF)[68]. Therefore, MSCs could provide trophic support to injured tissue by modifying the microenvironment to induce local precursor proliferation and differentiation, improving damaged tissue irrigation, and preventing parenchymal cell apoptosis[55,68]; and (6) MSCs are hypo-immunogenic[69], which represents the main advantage of MSCs over hematopoietic stem cells for clinical use, as histocompatibility between donor and receptor is not required and the recipients do not need to be conditioned before MSC transplantation[70]. Furthermore, MSCs have been administered to more than 1000 human patients with no evidence of adverse effects or tumor formation[70] (Table 1).

MSC TRANSPLANTATION: A PROMISING STRATEGY TO TREAT PATIENTS WITH ALD

Multiple mechanisms have been suggested to play a role in amelioration of liver diseases after MSC administration, such as: trans-differentiation of MSCs into hepatocytes, immunomodulation, inhibition of fibrosis development, protective effects on hepatic cells, and restoration of hepatic cell proliferation capacity (Figure 1).

Differentiation of MSC into parenchymal cells

The high degree of plasticity of MSCs has been widely described over the last decade or so[55,71]. Thus, MSCs have the potential cross the germ line barrier and differentiate into non-mesodermal cells (such as hepatocytes and neurons)[72]. It is important to note that MSC-derived hepatocytes need to not only express the genes found in mature liver cells, but express them at a level that is close to what is observed in the normal liver. Therefore, it is crucial to define which characteristics are needed for a differentiated cell to be comparable to a primary hepatocyte. The minimal set of functions of a true hepatocyte includes: (1) metabolic function (detoxification of xenobiotics and endogenous substances); (2) synthetic function (production of albumin, clotting factors, and complement); and (3) storage function (storage of glycogen and fat-soluble vitamins)[73].

Although the protocols for hepatocyte induction have been standardized for cultured MSCs[74,75], an organ-specific microenvironment is the most suitable place for them to differentiate into the required cell types. In this sense, Sato *et al*[76] were the first to demonstrate the *in vivo* hepatic differentiation

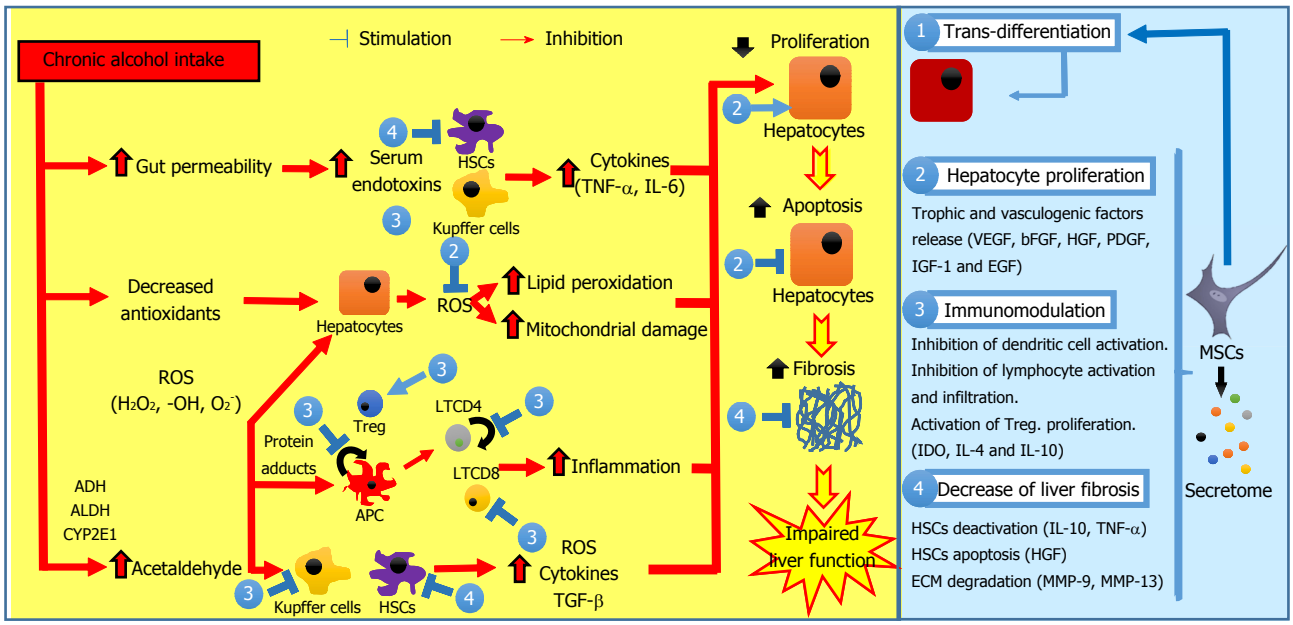

Figure 1 Pathogenesis of alcoholic liver disease and possible interventions of mesenchymal stem cells. Ethanol promotes the translocation of lipopolysaccharides from the gastrointestinal lumen to the portal vein. In Kupffer cells and in HSCs, lipopolysaccharides increase the expression of multiple pro-inflammatory cytokines, reducing liver regeneration. Chronic alcohol exposure reduces the intracellular concentration of antioxidants with subsequent mitochondrial dysfunction, leading to hepatocyte apoptosis. Acetaldehyde is highly toxic to hepatocytes because it binds to proteins forming adducts that promote glutathione depletion, lipid peroxidation, and mitochondrial damage. Additionally, these adducts act as antigens that activate the adaptive immune response, leading to lymphocyte recruitment to the liver. HSCs can be activated by damaged hepatocytes and activated Kupffer cells through various profibrogenic mediators, resulting in ECM accumulation and fibrosis. The interventions of MSCs include: (1) trans-differentiation into parenchymal cells; (2) induction of endogenous regeneration (*i.e.*, stimulation of hepatocyte proliferation, inhibition of hepatocyte apoptosis, and improvement of the impaired endogenous regeneration); (3) modulation of inflammation (*i.e.*, inhibition of APC maturation, proliferation, activation, and/or T-cell priming activity, reduction of lymphocyte proliferation and stimulation of Treg proliferation); and (4) decrease of liver fibrosis (*i.e.*, inhibition of HSC proliferation, stimulation of HSC apoptosis and induction of ECM degradation). APC: Antigen-presenting cell; ECM: Extracellular matrix; HSC: Hepatic stellate cell; MSC: Mesenchymal stem cell; ROS: Reactive oxygen species; TGF-β: Transforming growth factor-β.

potential of hMSCs. In this study, hMSCs were directly xenografted to the liver of allyl alcohol-treated rats, and they observed that some of the administered hMSCs differentiated into hepatocyte-like cells one month later. Additionally, the *in vivo* hepatic differentiation potential of MSCs has been demonstrated in rats[77], mice[78], sheep[79], and humans[51].

On the other hand, *in vitro* differentiated cells were found to express hepatocyte markers (alpha-fetoprotein, albumin, CK18, CK19, CYP1A1, CYP3A4, G6P, and human growth hormone-releasing factor)[80], store glycogen[81], clear ammonia and produce urea[82], and to secrete albumin and uptake low-density lipoprotein[83,84]. However, it is much more challenging to determine whether a cell is a true hepatocyte *in vivo*. Immunostaining for albumin, CK18, or hepatocyte nuclear factor are recognized indicators of hepatocyte trans-differentiation but not cellular functionality. It is important to note that differentiated MSCs still express mesenchymal markers such as CD90, α-smooth muscle actin, vimentin, and fibronectin, suggesting that complete trans-differentiation is not achieved[85].

Hepatic trans-differentiation potential is essential for MSCs-based therapies in the context of ALD, in which the injured hepatocyte cannot regenerate. However, the initial optimism has been tempered by the recognition of many groups that fusion of MSCs with endogenous hepatocytes is the main mechanism by which new hepatocytes are produced *in vivo*[86,87]. Hence, irrespective of whether the mechanism is MSC trans-differentiation or fusion, these events do not occur at a sufficiently high frequency to account for the observed functional improvement after MSC administration. Therefore, additional mechanisms may be involved in the regenerative process[88-90].

Modulation of inflammation by MSCs

Liver injury caused by persistent inflammation is accompanied with T cell, B cell and monocyte infiltration of the liver[91,92]. In this respect, MSC immunomodulatory and immunosuppressive properties could potentially be involved in the positive effects that MSC transplantation has in chronic and acute liver diseases.

MSCs regulate the activity of cells from both adaptive and innate immunity[93]. *In vitro*, they inhibit the differentiation of monocytic precursors into activated dendritic cells[94,95]. Thus, MSCs indirectly limit the cytotoxic expansion and activity of NK T lymphocytes[96]. Both *in vitro* and *in vivo*, MSCs downregulate the expression of proinflammatory molecules [interleukin (IL)-1β, IL-12, TNF-α, and interferon-γ] and secrete anti-inflammatory factors (IL-4 and IL-10), shifting the immune response pattern toward a protective Th2 type, and establishing a tolerogenic microenvironment where

activated T cells are unable to proliferate and die by apoptosis[97].

Another candidate for the suppressive effects of MSCs is indoleamine 2,3-dioxygenase, which is expressed by MSCs upon interferon-γ stimulation, leading to tryptophan depletion, and thus inhibition of T-cell proliferation[98]. This effect on T lymphocytes indirectly suppresses the function of B lymphocytes because their activation is mainly T-cell dependent. Moreover, MSCs can also modulate B-cell functions by inhibiting their proliferation, differentiation into antibody-secreting cells, and chemotaxis[99].

MSCs also promote the appearance of regulatory T cells, inducing antigen-specific tolerance[100]. Interestingly, it has been shown that the immunologic properties of undifferentiated MSCs are retained when they differentiate into parenchymal cells[101]. Therefore, both undifferentiated and differentiated MSCs will contribute to the maintenance of a microenvironment that allows tissue regeneration.

Induction of endogenous regeneration by MSCs

It is known that MSCs have the ability to secrete, *in vitro* and *in vivo,* a wide range of trophic factors, including VEGF, basic fibroblast growth factor, HGF, platelet-derived growth factor, TGF-β, IGF-1, and EGF[68]. The biologic effects of these factors can be both direct, by unleashing intracellular signalization pathways, and indirect, by inducing other cells from the microenvironment to secret additional bioactive factors. Therefore, it has been proposed that MSCs have a catalytic role in tissue regeneration, as once in the damaged tissue, they are able to modify the microenvironment by secreting factors that would: (1) prevent parenchymal cells from dying; (2) induce the proliferation and differentiation of endogenous progenitors; (3) promote neovascularization; and (4) avoid/revert fibrosis development[88,90].

Diverse studies have shown that < 1% of systemically administered MSCs are still present in any organ, including the lung, heart, kidneys, liver, spleen, and gut, one week after administration[102-104]. However, clinically, the beneficial effects associated with MSC administration can be observed for much longer than one week.

MSC-conditioned medium (MSC-CM) administration can recapitulate the beneficial effects of MSCs regarding tissue repair; for instance, data from van Poll *et al*[105] has provided the first clear evidence that MSC-CM procures trophic support for injured liver by inhibiting hepatocellular death and by stimulating liver regeneration. Although no specific mechanisms of action have been identified, soluble factors, including VEGF, HGF, IGF-1, EGF, IGF-binding protein, and IL-6, have been implicated in those regenerative effects.

Microvesicles (MVs) have recently been considered as important mediators of cell-to-cell communications, as they carry a complex load of proteins, lipids, mRNA, and microRNA, which might affect several cellular processes and pathways[106]. MVs account for approximately 10% of conditioned medium components in terms of protein amount; therefore, MSC-CM therapeutic activity could thus be partially attributed to MVs[107,108].

In addition to the induction of liver regeneration, antifibrotic properties of the MSC secretome have also been described. In this sense, Li *et al*[109] demonstrated that transplantation of MVs derived from human umbilical cord MSCs can alleviate liver fibrosis induced by carbon tetrachloride administration. These results have also been recapitulated by the administration of *ex vivo* expanded MSCs[109-112]. However, other studies have reported that MSCs can potentially be fibrogenic and contribute to increased fibrosis[113-115] or have no effect whatsoever[116,117].

These experimental results suggest two apparently contradictory scenarios; a great number of variables contribute to the inconsistences between the different observations. One such inconsistency is the difference in the properties of MSCs prepared in different laboratories, due to differences in the protocols used for MSC isolation and *ex vivo* expansion. There are also important differences between hMSCs and rodent MSCs, and even between different mouse strains[55]. Finally, another important factor is the dependence of the MSC differentiation process on most of the culture conditions or *in vivo* microenvironments, especially those developed in damaged tissue. In most of the cases, the signals that drive this differentiation process have not been characterized, so they cannot be replicated *in vitro*.

MSC TRANSPLANTATION IN ANIMAL MODELS OF LIVER INJURY

Numerous studies have tried to demonstrate the therapeutic potential of MSCs in the treatment of acute and chronic liver diseases, however, to date, a gap in the study of MSC administration for the treatment of ALD remains (Table 2). This gap is due, in part, to the lack of experimental animal models that recapitulate the full progression of ALD in human patients. Nonhuman primates are possibly the most similar model for human diseases[118,119]. For example, exposure of baboons to *ad libitum* alcohol intake leads to the progression of all stages of liver damage associated with ALD in humans. However, the relevance of nonhuman primates as a model of ALD is outweighed by the prodigious cost of maintaining them, which limits their utility to the field as a whole. Therefore, it is not surprising that the majority of alcohol research performed in animal models involves rodents[118,119]. The major disadvantage of rodent models with regard to experimental ALD is that the obtained liver pathology is limited predominantly to steatosis, with some necroinflammatory changes. The more-severe

Table 2 Preclinical studies using mesenchymal stem cells or their derivatives to treat liver injury

Model animal species	Liver injury induction/kind of liver injury	MSCs administration route	Number and source of transplanted MSCs	Therapeutic effect	Proposed mechanisms	Ref.
Rat	Allyl alcohol (ip administration)/ chronic damage	Intrahepatic	1×10^6 MSCs from human BM	Hepatocyte regeneration	Hepatocyte differentiation without evidence of cell fusion	[76]
Mouse	Low-level of radiation/ minimal, hepatic damage	Tail vein	2×10^4 MSCs from mouse BM	Hepatocyte regeneration	Hepatocyte differentiation	[78]
Mouse	Chronic exposure to high fat diet/ NASH	Tail vein	0.5×10^6 MSCs from mouse BM	Prevention of NASH onset Preclusion of the inflammatory process	Paracrine promotion of hepatic proliferation Increase in the fatty-acid oxidation enzymes expression	[110]
Mouse	Chronic exposure to atherogenic diet/NASH	Splenic capsule	0.1×10^6 MSCs from mouse adipose tissue	Restoration of albumin expression in hepatic parenchymal cells Amelioration of fibrosis Suppression of persistent hepatic inflammation	Modulation of inflammation Increase in MMP expression	[111]
Mouse	CCl_4 (ip administration)/ liver fibrosis	Spleen	0.5×10^6 MSCs from human amniotic membrane	Reduction of liver fibrosis Improvement of hepatic function	Inactivation of HSCs Reduction of hepatocyte apoptosis Promotion of liver regeneration Differentiation of hepatocyte-like cells	[126]
Mouse	CCl_4 (ip administration)/ liver fibrosis	Tail vein	0.5×10^6 MSCs from human BM	Reduction of liver fibrosis	Induction of MMP-9 expression Reduction in TGF-β expression	[121]
Rat	D-galactosamine (ip administration)/ fulminant hepatic failure	Penile vein	Conditioned medium from human BM MSCs	Reduction in the mortality rate Reduction in panlobular leukocyte infiltrates Reduction in hepatocellular death	Modulation of the immune response Trophic factor release (*i.e.*, VEGF, HGF, and IGF-BP)	[105,122]
Mouse	CCl_4 (ip administration)/ liver fibrosis	Intrahepatic	Exosomes derived from human umbilical cord MSCs	Recovery of serum aspartate aminotransferase activity Decrease in collagen type Ⅰ and Ⅲ, TGF-β1 level	Not determined	[109]

BM: Bone marrow; HGF: Hepatocyte growth factor; HSC: Hepatic stellate cell; IGF-BP: Insulin-like growth factor-binding protein; ip: Intraperitoneal; MMP: Matrix metalloproteinase; MSC: Mesenchymal stem cell; NASH: Nonalcoholic steatohepatitis; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor.

steatohepatitis and advanced liver damage observed in human patients (fibrosis and cirrhosis) is generally not observed in rodents[118,119].

Several *in vivo* studies have been performed to evaluate the therapeutic potential of MSCs in the context of liver fibrosis[54,56]. In most of the studies, liver fibrosis was induced by intraperitoneal or subcutaneous injection of carbon tetrachloride, however, this model cannot provide a perfect simulation of human etiology[120,121].

Application of MSCs in the *in vivo* models of liver fibrosis/cirrhosis ameliorates the development of the disease[54,56,111,112]. Similar results were obtained when MSC-CM or MVs were applied[105,108,109,122], suggesting that long-term survival of MSCs might not be necessary for their beneficial effects. In these studies, the reduction of fibrosis was correlated with the decrease in the synthesis of collagen Ⅰ and matrix metalloproteinase inhibitors, with a concomitant decrease in activated HSCs. Multiple mechanisms have been suggested to participate, such as immunomodulation[123], selective apoptosis of[124,125] or reversion to a quiescent state of HSCs, and production of protective factors[126,127].

Studies of *in vitro* co-cultures of MSCs with activated stellate cells have shown that, even in small numbers, MSCs can paracrinally inhibit the fibrogenic activity of activated stellate cells. This inhibition can be a consequence of the secretion of IL-10 and TNF-α by MSCs. Moreover, MSCs are able to induce apoptosis in reactive stellate cells, a process mediated in part by the secretion of HGF[125]. These results support the hypothesis that the therapeutic effects of MSCs on fibrosis inhibition are the result of the secretion of paracrine factors that modulate the proliferation, viability, and function of resident stellate cells. The production of matrix metalloproteinases can also be effective at reverting hepatic fibrosis. MSCs are capable of secreting and inducing the expression of matrix metalloproteinase-9 and -13 in other cells, the latter being the main rodent and human interstitial collagenase[128,129].

In ALD, as well as in more prominent cirrhotic liver, hepatocytes are reported to have reduced proliferative

capacity, which may reflect either the inhibitory effect of adjacent collagen Ⅰ or that they have reached replicative senescence after many rounds of injury and repair[44,45]. MSC infusion may increase the intrinsic ability of hepatocytes to proliferate by the release of proliferative trophic factors and cytokines, or by facilitating the breakdown of scar tissue, thereby removing a block to proliferation[130].

In our laboratory, we found that intravenous administration of bone marrow-derived MSCs into animals suffering from diet-induced metabolic syndrome and obesity restores liver function and avoids progression from steatosis to nonalcoholic-steatohepatitis[110]. Such MSC-mediated hepatoprotection was unrelated to metabolic syndrome reversion. Nevertheless, this has been associated with the potential of MSCs to enhance liver regeneration and/or manage the second hit required for the transition from steatosis to nonalcoholic-steatohepatitis, as an increased hepatic proliferation rate was found as well as an increased expression of fatty-acid oxidation enzymes. Thus, MSC administration could prevent the progression of ALD by reducing the impairment of fatty-acid oxidation.

Finally, the question of the ideal route of MSC injection remains one of the main unsolved issues regarding efficient administration of MSCs. Even if the tail vein seems to be the most often used administration route in animals, the portal vein and intrahepatic injections also seem to be efficient[129,131]. The optimal dose of cells or conditioned medium also needs to be evaluated because there are significant variations among studies in terms of the number of cells injected per animal.

CLINICAL TRIALS USING MSCS

MSCs have been successfully used in humans to treat different pathologies such as osteogenesis imperfecta[132], idiopathic aplastic anemia[133], graft-versus-host disease[134], and acute myelogenous leukemia[135]. Other applications have been to specifically avoid lung fibrosis injury after bleomycin challenge[136], and for the protection of cardiac function after a myocardial infarction[137]. In every case, clear therapeutic effects with no complications have been reported.

In the same direction, the translation of preclinical research on MSCs to clinical use for cirrhotic patients has generated great interest due to the growing population of patients with advanced liver diseases and the critical shortage of available liver donors.

To date, some clinical trials using hMSCs to treat patients with liver fibrosis have been published[112,138-145]. Unfortunately, in general, the studies were heterogeneous in their design and did not distinguish between the various etiologies of cirrhosis. ALD patients and viral hepatitis patients were mixed together in small case series.

Recently, Jang *et al*[140] evaluated the effect of autologous bone marrow-derived MSC transplantation on hepatic fibrosis in patients with alcoholic cirrhosis. After MSC administration, liver histologic improvements were observed in 6/11 patients, and recovery of liver function in 10 patients associated with a decreased expression of TGF-β1, collagen type Ⅰ, and α-smooth muscle actin, without significant complications or side effects during the study period[140]. These results support the use of these cells as a therapy for patients with alcoholic cirrhosis. However, further prospective, controlled studies are needed before MSC administration can be accepted as new strategy for antifibrosis therapy.

POTENTIAL LIMITATIONS TO CLINICAL TRANSLATION

Knowledge regarding MSC biology and their application in liver fibrosis treatment has significantly increased over the past years. Nevertheless, the clinical use of MSCs for liver regeneration, in particular ALD, is still in its beginnings, and fundamental questions remain to be addressed.

Although clinical trials have provided hope that MSCs could be a valuable resource for cell-based therapies for liver fibrosis, these results must be interpreted with some caution given the limited number of patients enrolled in each trial and the lack of appropriate controls. For example, patients with acute alcoholic hepatitis normally receive a high dose of prednisone therapy. However, the effect of high-dose steroids on the transplantation of MSCs is not well studied. There is some evidence that MSCs are glucocorticoid sensitive and are induced to differentiate into adipocytes with steroid exposure[146].

Clinical trials have shown that MSC-based therapy is relatively safe, and no serious detrimental effects have been reported in humans to date. However, some concerns have risen over the use of replicating cells, which may escape control as time elapses[147]. Some potential complications could also arise from intravascular administration of MSCs leading to vascular occlusion. Preclinical studies have not excluded the differentiation of injected MSCs into ectopic structures[148], myocardial calcification[149], and enhanced accumulation of fibroblasts and myofibroblasts in the lungs[150], as these events have been reported following MSC treatment.

CONCLUSION

Stem cell-based therapy represents a newly emerging therapeutic approach to treat ALD. MSCs are an attractive tool because they have been shown to trigger the regeneration of damaged liver tissue, with no evidence of significant adverse effects in either preclinical or clinical studies.

Due to the relation between pathologic events that occur in ALD development and the cellular and molecular mechanisms associated with MSC therapeutic effects, we believe that MSC transplantation could be a promising therapeutic strategy to manage ALD progression.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

Relationships among alcoholic liver disease, antioxidants, and antioxidant enzymes

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Abstract

Excessive consumption of alcoholic beverages is a serious cause of liver disease worldwide. The metabolism of ethanol generates reactive oxygen species, which play a significant role in the deterioration of alcoholic liver disease (ALD). Antioxidant phytochemicals, such as polyphenols, regulate the expression of ALD-associated proteins and peptides, namely, catalase, superoxide dismutase, glutathione, glutathione peroxidase, and glutathione reductase. These plant antioxidants have electrophilic activity and may induce antioxidant enzymes *via* the Kelch-like ECH-associated protein 1-NF-E2-related factor-2 pathway and antioxidant responsive elements. Furthermore, these antioxidants are reported to alleviate cell injury caused by oxidants or inflammatory cytokines. These phenomena are likely induced *via* the regulation of mitogen-activating protein kinase (MAPK) pathways by plant antioxidants, similar to preconditioning in ischemia-reperfusion models. Although the relationship between plant antioxidants and ALD has not been adequately investigated, plant antioxidants may be preventive for ALD because of their electrophilic and regulatory activities in the MAPK pathway.

Key words: Electrophile; Mitogen-activating protein kinase; Plant antioxidants; Reactive oxygen species; Preconditioning

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Core tip: The metabolic process of ethanol generates reactive oxygen species, which play a significant role in the deterioration of alcoholic liver disease (ALD). Antioxidant phytochemicals, such as polyphenols, upregulate the expression of antioxidant enzymes and peptides *via* the Kelch-like ECH-associated protein

1-NF-E2-related factor-2 pathway, which leads to antioxidant responsive elements in animal models. Furthermore, these antioxidants alleviate cell injury caused by oxidants or inflammatory cytokines *via* impairment of hyperactivation of mitogen-activating protein kinase pathways, similar to preconditioning in ischemia-reperfusion models. Although the relationship between plant antioxidants and ALD has not been adequately investigated, plant antioxidants may be preventive for ALD.

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INTRODUCTION

Humans are surrounded by many chemicals, including nutrients, phytochemicals, food additives, pharmaceuticals, and drugs. Although the intestine and liver absorb and metabolize many types of chemicals[1] for utilization or detoxification[2], some become more toxic once metabolized[3]. Ethanol, which is a component of alcoholic beverages, is one of the most common and abundant chemicals in daily life. Consuming ethanol can be relaxing and provides other benefits, but excessive drinking can be harmful physically and mentally and may decrease quality of life. Moderate consumption of alcohol has been shown to reduce the risks of cardiovascular disease[4] and non-alcohol fatty liver disease[5]. With moderate intake, most ethanol is oxidized by alcohol dehydrogenase and catabolized to acetaldehyde, which is subsequently catabolized to acetate *via* aldehyde dehydrogenase in the mitochondria. However, with binge drinking, ethanol is predominately metabolized to acetaldehyde *via* cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1), which comprises a microsomal ethanol-oxidizing system[6] that is involved in the generation of reactive oxygen species (ROS)[7-9]. Despite much evidence demonstrating a role for CYP2E1 in alcoholic liver disease (ALD), several of our studies have demonstrated that consumption of ethanol-containing diets significantly increased hepatic CYP2E1 levels without significantly affecting plasma alanine aminotransferase (ALT) activity (unpublished data). These findings support the existence of a potent endogenous antioxidant system that can prevent potential damage *via* the excessive expression of CYP2E1[10].

Binge drinking may cause liver injury, as demonstrated by increased blood levels of ALT, aspartate aminotransferase (AST), and/or lactate dehydrogenase (LDH)[11-14] and lipid accumulation in the liver-alcoholic fatty liver[12,13,15,16]. Hepatic functions are gradually lost with the progression of ALD[11], which is one of the most critical causes of cirrhosis[11,17]. Three mechanisms have been proposed to cause alcoholic liver injury: (1) acetaldehyde toxicity[18]; (2) metabolic generation of ROS or exposure to oxidative stress[10,19-21]; and (3) provocation of an immune response that causes oxidative stress in hepatocytes[13,22-24]. ALD patients appear to exhibit oxidative stress[11]; thus, increasing defense activities against this stress is important in the prevention of ALD.

In mammals, ROS is scavenged by antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and antioxidant substances, such as vitamins and glutathione (GSH) in collaboration with glutathione peroxidase (GPx) and glutathione reductase (GR)[25]. In previous studies, the induction and/or restoration of these substances and enzymes, which are reduced by ethanol administration, appeared to ameliorate ALD[12,13,23,26]. Some vitamins exhibit antioxidant activity and are reduced in the ALD model[27-29]. They are also deficient in ALD patients, although if present in sufficient quantities, may contribute to the prevention of oxidative stress[30]. Vitamin E is not only a lipophilic antioxidant but also may improve lipid metabolism *via* interaction with lipid accumulation-related proteins, namely patatin-like phospholipase domain containing 3 (PNPLA3) and microsomal triglyceride transfer protein[31]. However, several clinical studies have identified only partial effects of vitamin E in ALD[32,33]. Therefore, the induction of antioxidant enzymes may be more effective than vitamin supplementation in the prevention of ALD.

A trend in gastronomic culture is the exclusion of low molecular weight phytochemicals during plant breeding or processing because of their toxicity, taste, or deteriorating color. However, phytochemicals have recently received attention for their physiological activities in mammals. Many types of phytochemicals abundant in fruit and vegetables are known to have antioxidant activity. Although research efforts have focused on phenolic compounds due to their direct scavenging activity of ROS[34,35], their direct activity towards endogenous ROS appears limited in mammals because of their relatively low concentrations in the bloodstream[2,36,37]. However, many types of polyphenols, non-phenolic phytochemicals, and antioxidant-rich plant fractions have recently been reported to elicit an antioxidant defense system against liver damage induced by ethanol[34,35,38,39], other chemicals[40-43], or abnormal metabolism[21,44] to reduce oxidative stress and cell death[34,42,43,45] and to improve lipid metabolism[12,16,44,46] in various organs. In addition, some phytochemicals change both phase Ⅰ and phase Ⅱ enzymes of drug metabolism, including CYP2E1[7,13,16,47]. Recent reports indicated that some polyphenols can improve epithelial cell junctions[48-51], indicating a role for the hepatic immune response. These findings

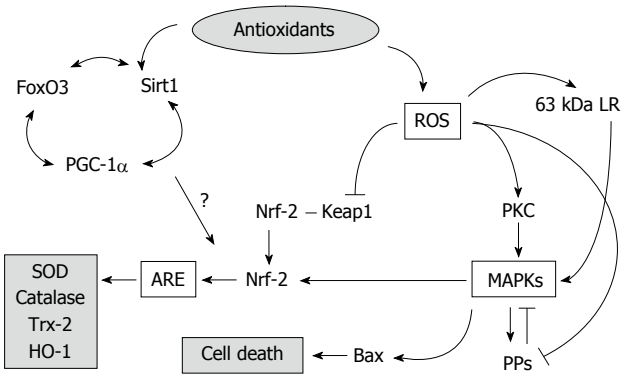

Figure 1 Oxidative stress-stimulating signaling pathways. The oval with the gray indicates the start point; gray boxes indicate consequences; other boxes indicate key substances. ARE: Antioxidant responsive element; FoxO3: Forkhead winged-helix box class O3 transcription factor; HO-1: Heme oxygenase-1; Keap1: Kelch-like ECH-associated protein 1; LR: Laminin receptor; MAPK: Mitogen-activating protein kinase; Nrf2: NF-E2-related factor-2; PGC-1α: Peroxisome proliferator-activated responsive element γ coactivator-1α; PKC: Protein kinase C; PP: Protein phosphatase; ROS: Reactive oxygen species; Sirt1: Sirtuin 1; SOD: Superoxide dismutase; Trx: Thioredoxin.

suggest that phytochemicals could potentially have a comprehensive preventive effect on ALD. However, the physiological activities of phytochemicals in the prevention of ALD have not been well recognized.

In this review, we discuss the physiological activities of phytochemicals and the mechanisms for cell injury, the regulation of antioxidant and pro-oxidant enzyme expression, and concomitant intestinal permeability. Herein, "antioxidants" are defined as the phytochemicals that elicit or enhance the antioxidant defense system, regardless of their radical scavenging activity. Because information regarding the effects of antioxidants in ALD patients or animal models is insufficient for discussion, various oxidative stress models in animals and cells are included. In particular, the mechanisms of non-alcoholic fatty liver disease (NAFLD) may comprise, in part, the mechanisms of ALD because these two diseases likely share many common pathways[31].

MECHANISMS OF LIVER INJURY FROM ALCOHOL CONSUMPTION

As a cause of oxidative stress, ROS are generated by pro-oxidant enzymes, such as CYP2E1 in hepatocytes[7,52,53] and NADPH oxidase (NOX) in Kupffer cells (liver-dwelling macrophages)[25]. In addition, populations of intestinal bacteria that comprise the intestinal environment have been suggested to be involved in ALD *via* stimulation of the immune system. For example, lipopolysaccharides (LPS) derived from intestinal bacteria[15,24,54] activate NOXs and produce inflammatory cytokines[55-58] in macrophages. Acetaldehyde increases the permeability of LPS between intestinal epithelial cells[15,59,60], which is also involved in the deterioration of ALD. Dietary polyunsaturated fatty acids are also thought to enhance oxidative stress[15,29] and are a source of prostaglandins[61]. In a previous study, ethanol administration increased the plasma prostaglandin E_2 level[62], and some prostaglandins are thought to cause inflammation in NAFLD[61,63]. These data suggest that prostaglandins enhance deterioration of ALD; however, the influence of antioxidants on prostaglandins will not be detailed here.

As shown in Figure 1, oxidative stress stimulates intracellular events *via* the mitogen-activating protein kinase (MAPK)[64] pathway, as initiated by the activation of protein kinase C (PKC)[30,65,66] or the degradation of protein phosphatases (PPs)[67]. These signals activate the Kelch-like ECH-associated protein 1 (Keap1)-NF-E2-related factor-2 (Nrf2) pathway, which leads to antioxidant responsive element (ARE)[45,68-70]. However, MAPK hyperactivation also leads to cell death *via* activation of the Bax/Bcl-2 pathway[71,72]. In addition, antioxidant enzymes have been reported to be induced *via* several intracellular pathways, such as the Keap1-Nrf2-ARE pathway[45,69,70,73] and the Sirt1 (sirtuin-1)-FoxO3 (forkhead winged-helix box class O3 transcription factor)-PGC-1a (PPARγ coactivator-1a) pathway[45,68]. The regulation of Sirt1 and Nrf2 levels has also been reported[45], which implies cross-talk between both pathways, whereas the activation of Sirt1 and resveratrol, an activator of Sirt1, have been reported to inhibit the DNA-binding activity of Nrf2 *via* deacetylation *in vitro*[74]. Taken together, substances that deactivate or normalize MAPKs and/or activate ARE or Sirt1[45,75] are potential candidates for the prevention of ALD, but the mechanisms are unknown.

Antioxidant enzymes and peptides

In mammals, SOD generates hydrogen peroxide, which is catabolized to a hydroxyl radical by catalase and detoxified by GSH in collaboration with GPx[25]. The oxidized glutathione form is recruited to GSH by GR with NAD(P)H[76]. Heme oxygenase-1 (HO-1) contributes to the antioxidant system because of the production of bilirubin as a redox substance.

It has been suggested that the hepatic catalase level is negatively associated with the severity of alcoholic liver injury[10] and that SODs scavenge hydroxyl peroxides generated in the cytosol and mitochondria, thereby terminating autoxidation. Thus, catalase and SODs are essential for the antioxidant system. There are three isozymes of SOD in the cytosol, mitochondria, and extracellular matrix: CuZn-SOD, Mn-SOD, and extracellular SOD. SOD levels have been shown to be regulated by MAPK activity[77]. GSH is not an enzyme but a redox tripeptide that acts as a proton donor. GSH levels, GPx content, and/or GR content were reduced in rats fed ethanol diets and, in some cases, ALD animals[16,23,62] or under other oxidative conditions[3,78]. The FoxO transcriptional factor is involved in GPx and Sirt1 protein expression[79]. These findings indicate that in addition to catalase and SOD, GSH is essential for

reducing hepatic oxidative stress.

Under oxidative conditions, HO-1 appears to be rapidly induced *via* the Keap1-Nrf2 pathway[45,69,80,81]. This enzyme may also be involved in the immune response[55]. Furthermore, in ALD model animals, HO-1 levels have been reported to be reduced[13,16,82]. Adiponectin has received recent attention because of its anti-inflammatory functions *via* Sirt1 activation, HO-1 induction, and NOX suppression in Kupffer cells[55]. However, the blood concentration of this adipokine was higher in ALD patients compared with controls[83] or equal to the controls in ALD animals[84], which suggests that adiponectin may be less effective against ALD than antioxidants.

Thioredoxin (Trx) is a ubiquitous scavenger of oxidative species. Endogenous Trx is reported to be reduced by ethanol ingestion; however, the levels can be restored by supplementation with exogenous Trx, which has been demonstrated to ameliorate the symptoms of ALD[84]. Because Trx is a peptide, it must be digested in the digestive system, indicating that it is difficult for exogenous Trx to directly scavenge hepatic ROS.

Pro-oxidant enzymes

In microsomes, CYP2E1 is a phase Ⅰ enzyme of drug metabolism that adds a hydroxyl residue to chemicals to increase hydrophilia and may generate ROS[7-9]. Chronic ingestion of ethanol and other small chemicals increase hepatic CYP2E1. CYP2E1 induction has also been demonstrated in animals with NAFLD[52,85] and hepatic insufficiency. Insulin signaling may suppress CYP2E1 expression[53] *via* the Akt pathway but not the MAPK pathway[86], with subsequent expression of certain microRNAs[87].

Macrophage-like cells, including Kupffer cells, express NOXs and generate ROS with the consumption of NAD(P)H[24] to eliminate xenobiotics[25]. Many isoforms of NOXs have been identified, and NOX-2 is uniquely expressed in phagocytes. NOX expression was regulated *via* the Keap1-Nrf2 pathway in a mouse glial-neural co-cultured system[88] in which NOX-2 predominantly caused oxidative stress. In ALD animals, NOX-2 in Kupffer cells was activated by LPS[55]. In addition, Kupffer cells produce inflammatory cytokines[13,24,55], such as tumor necrosis factor alpha (TNF-α) and interleukin-6. Thus, the reduction of NOXs and inflammatory cytokines are important for ALD.

Given the gut-liver axis in ALD, intestinal conditions play a considerable role in ALD severity, particularly conditions mediated by LPS[15,60]. In the large intestine in humans (or the cecum in animals), an enormous number of intestinal bacteria live and ferment undigested food matter, flaked epithelial cells, and digestive fluid[25]; some of these species generate LPS, which provokes the host's immune system[15]. Small amounts of LPS can pass through gaps in the epithelial cells into the intestine. Ethanol or its metabolites are reported to widen this gap[15,59]. Therefore, improving intercellular junctions or reducing LPS-producing bacteria may have a partial preventive effect on ALD[15].

PLANT ANTIOXIDANTS

Classification of plant antioxidants

Figure 2 shows the structures of representative antioxidants abundant in fruit and vegetables. Polyphenol is a generic name for compounds that have a mono- or polycyclic structure with hydroxyl residues. Flavonoids, including anthocyanins, catechins, and flavonols, form one of the largest groups of polyphenols. Anthocyanins have a red, purple, or blue color in grapes[42], berries[34], seed coats[89], and root crops[37,77]. Catechins include epicatechin, epigallocatechin, and epigallocatechin galate (EGCG) and are sometimes referred to as "tannins"[35]. Proanthocyanidins are polymers of catechins (but not anthocyanin despite the similarity in names); they are categorized as catechins and are widely abundant in crops, particularly tea[27,90], apples[91], and grapes[92]. Quercetin, kaempferol, and isorhamnetin belong to the flavonol group and are ubiquitous in plants. Narirutin and hesperidin belong to the flavanone group and are abundant in the albedo of citrus peel[14,23]. Resveratrol is categorized as a stilbenoid, a phytoalexin, and is present in wine[93] and grapes[42]; it has recently received substantial attention for its physiological functions. Chlorogenic acid is a caffeic acid derivative and one of the most widely consumed polyphenols because of its abundance in coffee and other plants. Alkaloids, such as berberine[46], are also included in the polyphenol group. Curcumin, a curcuminoid present in turmeric, has a yellow color and also belongs to polyphenols.

Lignans, a terpenoid whose metabolites exert estrogenic activity in the lumen, as well as isoflavones and coumestans possess antioxidant activity. Sulfide and thiocyanate compounds are present in garlic[12,82], onions[47], and *Brassicaceae* plants[16] and are reported to be chemopreventive.

PROVOCATION OF THE ANTIOXIDANT SYSTEM BY PLANT ANTIOXIDANTS AND PLANT EXTRACTS

Flavonoids

In animal models, quercetin ameliorated lipid metabolism and ethanol-induced liver damage by inducing antioxidant enzymes, increasing GSH levels, and reducing CYP2E1 activity[20,39]. Quercetin also inhibited the activity and expression of CYP2E1 in human hepatocytes[20,94], which was consistent with *in vivo* findings. In non-alcoholic steatohepatitis animals, quercetin ingestion increased hepatic catalase, SOD, GPx, and GR activities and the GSH level[21] and reduced hepatic lipid accumulation and CYP2E1

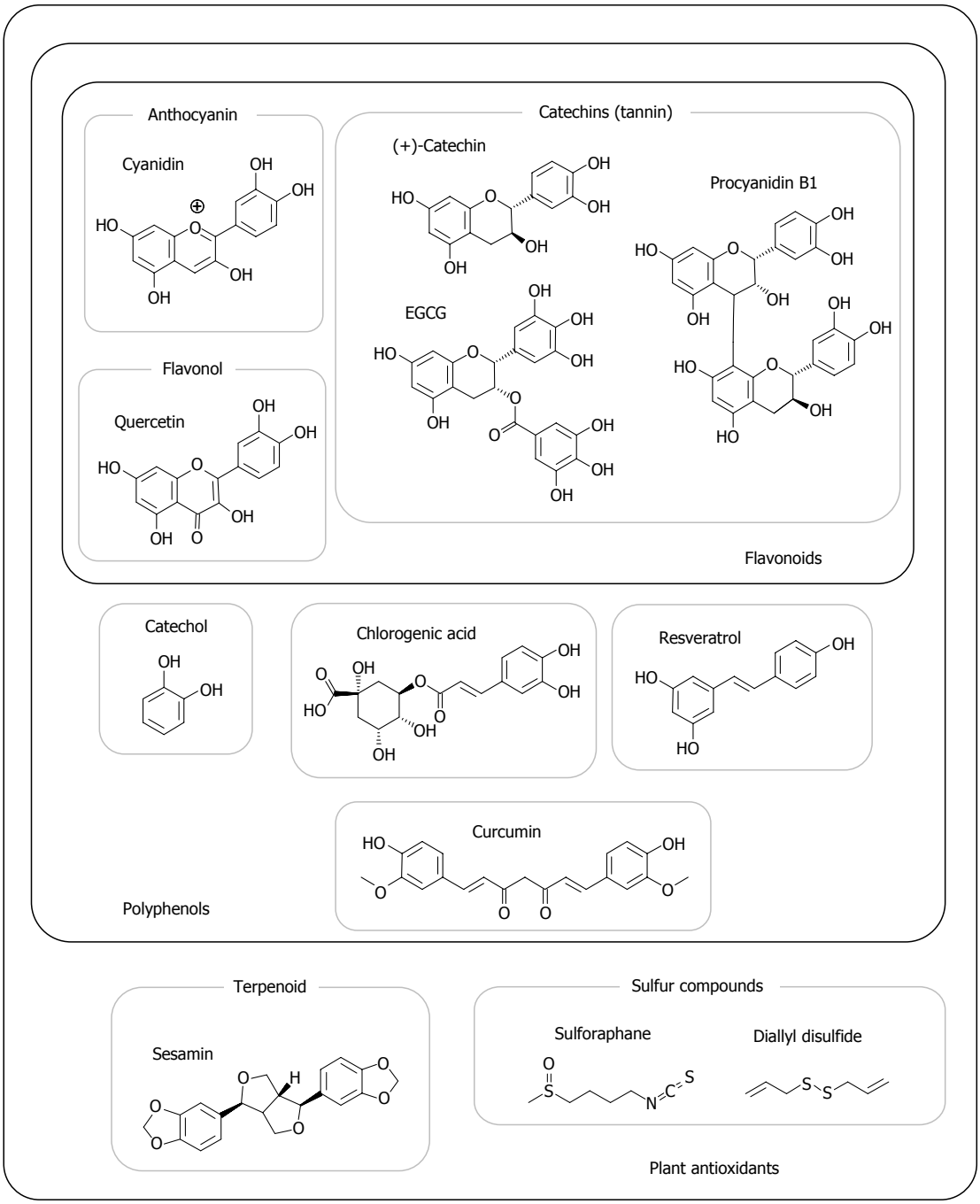

Figure 2 Structures of representative plant antioxidants and their classification.

expression[21,85]. A computer simulation predicted the involvement of quercetin in PGC1a and PNPLA3[31]. Furthermore, hyperoside (quercetin-3-O-galactoside) has been reported to increase cell viability and HO-1 activity *via* MAPKs and ARE[95] in L-02 cells.

Pigments from grapes[42], colored potatoes[77], and black soybean seed coats[89] that contain abundant anthocyanin have been reported to induce antioxidant enzymes *via* the alteration of MAPK activities in cells in other oxidative conditions. An anthocyanin fraction from bilberries appears effective in improving lipid metabolism *via* the AMP-activated protein kinase pathway[96]; however, its involvement in ALD has not been assessed. Alcohol-free red wine increased the blood antioxidant capacity in a human study[97], which suggests a preventive function of the polyphenol fraction in red wine against ALD. However, other studies have demonstrated that alcohol-free red wine worked with ochratoxin A to increase the intercellular permeability in Caco-2/TC7 cells[98], and alcohol-containing red wine increased hepatic and renal CYP2E1 expression in rats, whereas ethanol did not[99]. Malvidin, an anthocyanin in red wine, has been reported to attenuate MAPK activity, which was

promoted by LPS[64], and to enhance PP activity in RAW 264.7 macrophage cells. An anthocyanin-rich extract from colored potato increased Mn-SOD expression *via* extracellular signal regulated kinase (ERK) activation in HepG2 cells[77]. It has also been reported that an ethanol-induced acute gastric lesion was prevented by the ingestion of strawberry extract rich in anthocyanin prior to ethanol treatment *via* the induction of gastric antioxidant enzymes[34].

In animal studies, catechin- and tannin-rich extracts from pecan nut shells improved ALD symptoms by restoring antioxidant enzymes[35,38]. A tea extract rich in catechins reduced CYP2E1 expression and hepatic lesion *via* paracetamol injection[92], and a diet that contained EGCG improved hepatic injury; although there was no reduction in hepatic CYP2E1 levels[100]. In a clinical study, EGCG-rich green tea and its extract also increased the blood GSH level[90]. The ingestion of green tea extract also restored antioxidant activity in the brain that had been decreased by ethanol and aging[28]. Furthermore, catechins have been reported to suppress the expression of NOX and inflammatory cytokines in macrophages[56], dendrocytes[57], and human cerebral microvascular endothelial cells (hCMEC)[101] as well as restore antioxidant enzymes in human neuroblastoma cells[102]. Catechins have both antioxidant and pro-oxidant activities. They have recently been reported to stimulate the 63 kDa laminin receptor[56,57,101,103], which ROS may initiate[104], and consequently to calm over-activation of the immune system *via* the inactivation of the Toll-like receptor (TLR) 2 and 4 pathways. TLR 4, in particular, plays a central role in Kupffer cell stimulation with LPS and the induction of ALD deterioration[57]. Dietary catechins may thus contribute to the impairment of ROS generation *via* LPS and the prevention of ALD.

Citrus flavonoids, narirutin, and glycosylated citrus flavonoids also improved ALD and reduced inflammatory cytokine levels[14,23].

Other phenolic antioxidants and non-phenolic antioxidants

Resveratrol (Figure 2) restores or induces antioxidant enzymes in ALD model rats[93], lung fibroblasts[105], and rats with spontaneous hypertension[75] and diabetes[44,73] *via* the activation of sirtuins in some cases. *In vitro*, resveratrol stimulated HO-1 induction *via* the MAPK-Nrf2 pathway in PC12 cells[81]. Thus, red wine consumption is likely to be superior to other alcoholic beverages in the prevention of ALD. Resveratrol concentrations in wine may be insufficient to prevent ALD; however, it may be responsible for the "French paradox"[106]. Resveratrol has been reported to activate monocytes and produce inflammatory cytokines *in vitro*, which indicates that provoking the immune system with resveratrol may not prevent the deterioration of ALD[107]. Thus, excessive red wine consumption should not be recommended. Polydatin, a resveratrol glycoside, stimulates Sirt1 and Nrf2 and induces antioxidant enzymes in glomerular cells[45].

Chlorogenic acid (Figure 2) and caffeic acid restored the hepatic activity of SOD and GPx and hepatic injuries promoted by methamphetamine injection for 7 d[43].

Honokiol, identified in *Magnolia officinalis*[19], improved ALD, restored the hepatic GSH content and SOD activity, and reduced inflammatory cytokine levels in an ALD animal model[19].

Hispidin, a fungal polyphenol with PKC-inhibitory activity, increased HO-1 and catalase activities in H9c2 cardiomyoblast cells[65].

Berberine is a benzyl isoquinoline alkaloid in the *Coptis* genus that has been reported to reduce ALD symptoms, increase levels of GSH and PGC1a, and normalize CYP2E1 expression in the livers of animals fed an alcohol-containing diet[46].

The sulfur-containing compounds (Figure 2) diallyl disulfide and garlic oil have been reported to improve alcoholic hepatic injury[12] by increasing HO-1 levels *via* the Nrf2 pathway and increasing the GSH level *in vivo*[82] and *in vitro*[94]. A similar preventive effect has also been identified in diallyl sulfide treatment in astrocytes[30]. Sulforaphane has been reported to act as an inducer of HO-1[16], which suggests that these compounds may be useful in the treatment of ALD. In addition to restoring HO-1 levels, sulforaphane improved hepatic lipid accumulation in ALD animals[16]. The consumption of onion powder, which is rich in sulfide compounds and flavonols, has also been reported to reduce hepatic CYP2E1 levels in normal rats[47].

Oleanolic acid, a triterpenoid, restored antioxidant enzymes and increased nucleic Nrf2 levels and improved ALD[13]. Sesamin (Figure 2) is a well-characterized terpenoid in sesame seeds that may contribute to the reduction of fatty liver by promoting β-oxidation of fatty acids and inducing hepatic aldehyde dehydrogenase[108,109]. Maslinic acid, a triterpenoid rich in basil, brown mustard, and other plants, has been reported to protect hepatic injury *via* acute ethanol toxicity[62]. These data suggest that some types of terpenoids may improve the symptoms of ALD.

Curcumin (Figure 2), but not resveratrol, has been reported to restore hepatic antioxidant enzymes reduced by aflatoxin in rats[110]. Curcumin also increased antioxidant enzymes as well as Nrf2 and HO-1 levels in quails under heat stress[111].

Mangiferin, identified in mango[112], is a xanthine derivative that has been reported to restore pulmonic and hepatic antioxidant enzyme levels reduced by benzo(a)pyrene in mice[3].

Plant extracts that contain significant amounts of antioxidants also prevent oxidative damage in various other organs. An extract from black tea[27] improved ALD symptoms in rats. The extracts from apples[91], *Amorphophallus commutatus*[40], cinnamon[113], and hibiscus[22,41] partially normalized hepatic oxidative

stress induced by chemical toxins.

Improvement of fatty acid accumulation

Alcoholic fatty liver is a predictive symptom of ALD, and hepatic inflammation is also present in non-alcohol steatohepatitic animals[21,41,52]. Moreover, a computer simulation predicted many common pathways between alcoholic fatty liver and NAFLD that were associated with inflammation, lipid metabolism, and some immunity[31]. These data suggest that a reduction in lipids in the liver may lead to an improvement in liver injuries[16,19,100]. In addition to the induction of antioxidant enzymes, some plant antioxidants have recently been reported to improve lipid metabolism and reduce hepatic lipid accumulation[19,39,46], which may also contribute to the amelioration of ALD.

Improvement of intestinal permeability by plant antioxidants and plant extracts

Antioxidants, such as quercetin, resveratrol, EGCG, and naringenin, prevent the downregulation of junction proteins, namely, Zo-1 and/or Occludins, and consequently enhance intercellular barrier functions *in vitro*[49] and *in vivo*[50]. In contrast, EGCG has been reported to disturb the barrier function of hepatic epithelial cells[114] because of ROS-induced ERK activation. In addition to intestinal cell models, cocoa polyphenol extract improved barrier functions disturbed by a high glucose condition in retinal pigment epithelium cells[51]. Cocoa polyphenol extract and resveratrol also attenuated the permeability of renal cell junctions *in vitro*[48,115], and EGCG increased the adhesion of hCMEC[101]. The tightness of cellular junctions regulated by antioxidants may be involved in the severity of ALD and should be elucidated.

Mechanisms for ALD prevention via plant antioxidants

Cellular oxidative stress is caused by many factors, such as exposure to humoral factors[22,75], enzymatic generation of ROS[7-9,24], metabolites of chemicals[41,91,102,116], or the mitochondrial respiratory chain[39]. Two major mechanisms may be proposed for hepatic injury prevention *via* oxidation: (1) the impairment of oxidative signaling that leads to cell death; and (2) the activation of the Keap1-Nrf2 pathway, which results in the induction of antioxidant enzymes.

As a leading mechanism, "preconditioning" in ischemia-reperfusion models has been proposed to alleviate tissue damage. In ischemia-reperfusion models, excessive ROS are present following reperfusion, whereas slight ischemic-reperfusion pretreatment to tissues or cells alters MAPK activities and interferes with cellular damage[117-119]. It has been reported that ROS stimulate PKC, MAPKs, and subsequent events that lead to cell death[89] or induce an antioxidant system (Figure 1). MAPKs appear to activate both PPs[66,120] and Nrf2[69]. Once activated, PPs may deactivate not only MAPKs but also other phosphorylated proteins related to the MAPK signaling pathways[66], which may lead to a comprehensive impairment of MAPK signaling. Despite their antioxidant activity, polyphenols also have a slight pro-oxidant activity[72,121]. This impact may increase MAPK and PP activity[103] or PP stability[120] prior to crucial oxidative stress by ROS. At minimum, PPs activated by antioxidants may partially inhibit MAPK pathway activation. Following pretreatment with plant antioxidants, the hyperactivation of MAPKs by injuring stimuli appears to decrease[22,41,48,64]. These findings may support the preconditioning hypothesis[1]. Taken together, ROS and/or MAPK are key regulators of both cell injury and antioxidant enzyme induction.

In addition, this mechanism can explain the effects of antioxidants on the barrier functions of epithelial cells. Junction proteins and the intercellular barrier function are disturbed by oxidative stress[48,114]. Antioxidants have been reported to exhibit minimal activity to generate ROS[114,121] and subsequently activate MAPKs, which disturbs barrier function *in vitro*[114]. However, antioxidant pretreatment may diminish excessive oxidative stress, as previously discussed, which leads to the protection of barrier function[49,50].

It has been suggested that ROS (and electrophilic reagents) directly activate the Keap1-Nrf2 pathway. Keap1 is a sensor of intracellular oxidative stress and couples with Nrf2[122]. Once Keap1 is oxidized, Nrf2 is released, moves to the nuclei, and activates ARE. Regarding the relationship between chemical structures and antioxidant activities, it has been suggested that electrophilic compounds, such as flavonoids, curcumin, and thiocyanate-related compounds, stimulate the Keap1-Nrf2 pathway[122]. Satoh *et al*[123] proposed the importance of ortho- or para-positions of hydroxyl residues in the benzene structure, which result in hydroquinone and catechol, respectively (Figure 2), because of their electrophilic residue. Some flavonoid compounds have a catechol structure (Figure 2), which indicates an interaction between flavonoids and Keap1. These results may support the hypothesis proposed by Satoh *et al*[123].

This hypothesis suggests that antioxidants directly activate Keap1. However, some antioxidants appear to induce antioxidant enzymes *via* MAPK activation despite the upper proteins of Keap1 (Figure 1), as demonstrated with specific inhibitors of MAPKs that diminished the induction[77] or activation of Nrf2[81]. Antioxidants may contribute to the induction of antioxidant enzymes *via* MAPK pathways rather than through direct activation of Keap1. Moreover, resveratrol has a resorcinol structure rather than a catechol structure. Resorcinol has less electrophilic activity than catechol[123]; however, it appears to stimulate Nrf2[122]. This mechanism must also be elucidated.

In *in vivo* studies, the ingestion of antioxidants induces (or tends to induce) antioxidant enzymes in the lung[3], thymus[124], brain[28,125], and kidney[45], despite very low concentrations in the bloodstream[2,36,37].

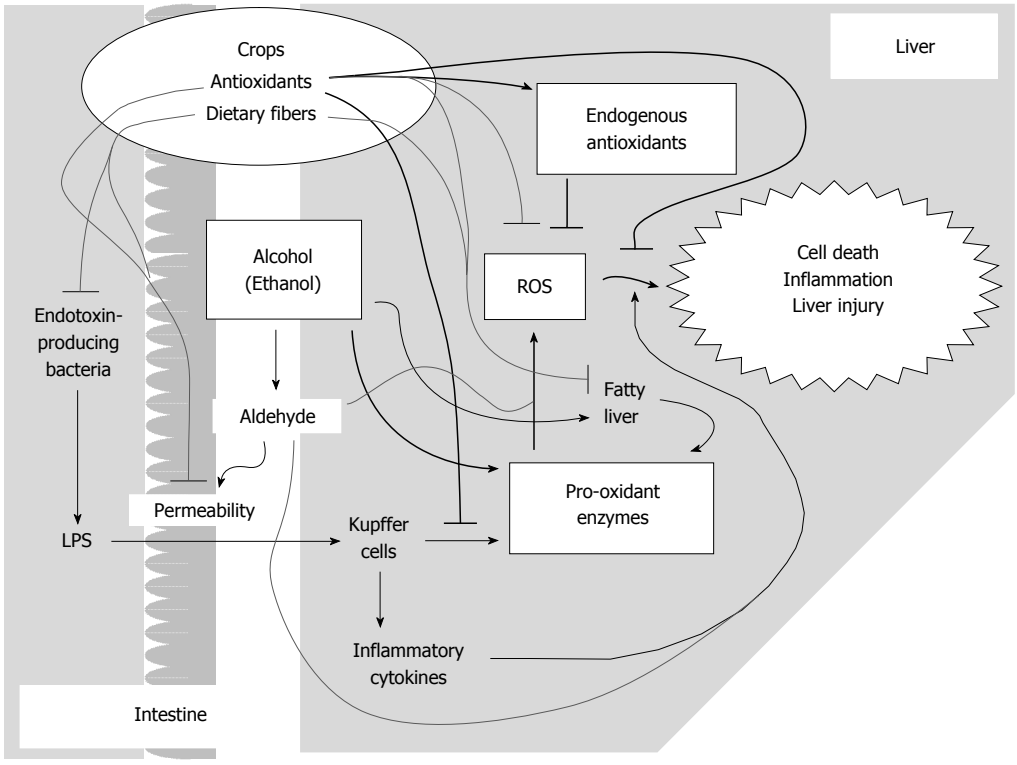

Figure 3 Potential multiple effects of crop components on alcoholic liver disease. LPS: Lipopolysaccharide; ROS: Reactive oxygen species.

These reports imply that there is an intermediate signal by polyphenols, such as nerve and/or humoral pathways, rather than direct stimulation of cells or organs; they may also be explained by remote ischemic preconditioning[117]. This preconditioning suggests that some types of stimuli can regulate MAPK activities in remote organs.

PERSPECTIVE

Even ubiquitous plant antioxidants, such as anthocyanins and flavonols, appear to have many physiological activities, indicating that botanical substances can provoke the antioxidant system. Apart from oxidative stress *via* lipid accumulation, lipids also appear to be a central cause of ALD. For example, prostaglandins, which are initiated by phospholipase (PL) A_2 and activated by cyclooxygenases[61], are involved in inflammatory events, and PNPLA3 has been suggested to have PLA_2 activity and to regulate hepatic lipid accumulation[63]. Therefore, the regulation of prostaglandins and/or expression of their related proteins may be critical for the improvement of ALD.

Fruits and vegetables are great sources of antioxidants as well as dietary fibers (DFs)[126], which were once considered to be unwanted materials or non-nutrients. It is now well established that the ingestion of DFs improves lipid metabolism and reduces hepatic lipids[127,128]. Some types of DFs, particularly water-soluble fibers, promote the excretion of lipids into feces and the synthesis of short-chain fatty acids (SCFA) in the intestine[126,129], which are proposed as prebiotics. Oral ingestion of butyrate, a type of SCFA produced from DF, promotes junction protein expression and an increase in intestinal barrier function[130]. These findings also suggest the potential of DFs in the prevention of ALD. Thus, intact fruits and vegetables, including both antioxidants and DF, are worthy of consideration for ALD prevention.

Mammals often intrinsically treat plant chemicals as xenobiotics and have developed metabolic systems against phytochemicals[1]. The human body evolved with environmental factors, including phytochemicals and DFs. The data reviewed here imply the necessity for the unwanted materials to elicit an accomplished defense system, a barrier function in the intestine and a chemical metabolizing system in the intestine, and liver against xenobiotic substances.

However, most of these data are derived from animal and cell studies. In these studies, antioxidants may, in some cases, be overdosed[75], which makes it difficult to justify their effectiveness in humans, particularly ALD patients who may have impaired liver functions[11]. As previously reported, vitamin E supplementation only partially improved ALD[32,33] despite its effectiveness in cell studies. Thus, it is important for future studies to accumulate clinical data regarding the relationships among ALD, antioxidants, and antioxidant enzymes.

In conclusion, plants have a potential role in the prevention of ALD (Figure 3). Although most individuals are aware that abstinence from alcohol is the most

effective way to prevent ALD, it is recognized that this is not easy. Therefore, it is important to improve our defense system against ALD. Many types of plant antioxidants with electrophilic activity may activate antioxidant enzymes or peptides under oxidative conditions and alleviate ALD, which may occur *via* a mechanism that is somewhat similar to preconditioning in ischemia-reperfusion models[117-119]. The antioxidants reviewed here are common in vegetables and fruits, which can be easily consumed. Moreover, plants contain abundant amounts of DF and vitamins. Vitamins are wasted by binge drinking[27,28], and DFs can improve lipid metabolism and intestinal conditions[127,128] in mammals. Therefore, non-processed food materials may have considerable intrinsic potential. Clearly, ALD patients should be administered appropriate medications to facilitate recovery from crucial damage. However, fresh vegetables and fruits may be more effective than processed foods in comprehensively preventing hepatic damage induced by alcohol. Antioxidants commonly taste bitter, and DFs appear to exhibit a bad texture; thus, they have been eliminated from foods over centuries. However, humans have evolved alongside phytochemicals and DFs to overcome these issues. Thus, an approach that elicits the intrinsic potential of the human body to prevent ALD and other lifestyle-related disorders should be reconsidered.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

Metabolic derivatives of alcohol and the molecular culprits of fibro-hepatocarcinogenesis: Allies or enemies?

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Abstract

Chronic intake of alcohol undoubtedly overwhelms the structural and functional capacity of the liver by initiating complex pathological events characterized by steatosis, steatohepatitis, hepatic fibrosis and cirrhosis. Subsequently, these initial pathological events are sustained and ushered into a more complex and progressive liver disease, increasing the risk of fibro-hepatocarcinogenesis. These coordinated pathological events mainly result from buildup of toxic metabolic derivatives of alcohol including but not limited to acetaldehyde (AA), malondialdehyde (MDA), CYP2E1-generated reactive oxygen species, alcohol-induced gut-derived lipopolysaccharide, AA/MDA protein and DNA adducts. The metabolic derivatives of alcohol together with other comorbidity factors, including hepatitis B and C viral infections, dysregulated iron metabolism, abuse of antibiotics, schistosomiasis, toxic drug metabolites, autoimmune disease and other non-specific factors, have been shown to underlie liver diseases. In view of the multiple etiology of liver diseases, attempts to delineate the mechanism by which each etiological factor causes liver disease has always proved cumbersome if not impossible. In the case of alcoholic liver disease (ALD), it is even more cumbersome and complicated as a result of the many toxic metabolic derivatives of alcohol with their varying liver-specific toxicities. In spite of all these hurdles, researchers and experts in hepatology have strived to expand knowledge and scientific discourse, particularly on ALD and its associated complications through the medium of scientific research, reviews and commentaries. Nonetheless, the molecular

mechanisms underpinning ALD, particularly those underlying toxic effects of metabolic derivatives of alcohol on parenchymal and non-parenchymal hepatic cells leading to increased risk of alcohol-induced fibro-hepatocarcinogenesis, are still incompletely elucidated. In this review, we examined published scientific findings on how alcohol and its metabolic derivatives mount cellular attack on each hepatic cell and the underlying molecular mechanisms leading to disruption of core hepatic homeostatic functions which probably set the stage for the initiation and progression of ALD to fibro-hepatocarcinogenesis. We also brought to sharp focus, the complex and integrative role of transforming growth factor beta/small mothers against decapentaplegic/plasminogen activator inhibitor-1 and the mitogen activated protein kinase signaling nexus as well as their cross-signaling with toll-like receptor-mediated gut-dependent signaling pathways implicated in ALD and fibro-hepatocarcinogenesis. Looking into the future, it is hoped that these deliberations may stimulate new research directions on this topic and shape not only therapeutic approaches but also models for studying ALD and fibro-hepatocarcinogenesis.

Key words: Alcoholic hepatitis; Lipopolysaccharide; Fibro-hepatocarcinogenesis; Mitogen activated protein kinase; Transforming growth factor beta; Small mother against decapentaplegic

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Core tip: Alcoholic liver disease (ALD) leading to fibro-hepatocarcinogenesis may show a bidirectional origin within the gut-liver axis. We bring to light the subtle reprogramming of the gut epithelium, gut microbiome and hepatic cells by both metabolic derivatives and unstable chemical species secondary to chronic alcohol intake, and their concerted role in ALD. We specifically highlight the integrative role of transforming growth factor-β/Smad, which synchronizes inflammatory and fibrogenic signals within the gut-liver axis. The gut may provide a less invasive option not only for prognosis and treatment of ALD but also for future research. We suggest that therapies for ALD and fibro-hepatocarcinogenesis should focus on restoring the gut microbiome.

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INTRODUCTION

It is a common knowledge that continuous heavy alcohol intake (80 g/d by men and 40 g/d by women) spanning several years (10-20 years)[1-3] may ultimately lead to chronic liver injury most often characterized by steatosis, steatohepatitis, liver fibrosis and cirrhosis leading to increased risk of fibro-hepatocarcinogenesis[1,4-6]. Many reviews[6-9] and research reports[10-12], just to mention but a few, have all emphasized the pathological role of alcohol and its metabolic derivatives in ALD as well as efforts to identify some of the signaling pathways crucial in alcohol-induced liver disease. These important expert inputs have provided new insights in our understanding of ALD and fibro-hepatocarcinogenesis and have also provided new research directions about these diseases. Nevertheless, the pathological and molecular signaling pathways which underpin the initiation and progression of alcohol-induced liver injury leading to fibro-hepatocarcinogenesis still remain incompletely elucidated. For instance, signaling pathways that integrate gut-dependent alcohol-induced dysbiosis, inflammation and liver-specific alcohol-related inflammation, immune regulation and fibrogenic signals have so far remained elusive. The current difficulty in elucidating the molecular pathogenesis of alcohol-induced liver disease is multifaceted. (1) The anatomical position of the liver coupled with the diversity of agents in terms of number, biochemical properties, physicochemical properties, toxicity potential, their duration/frequency of exposure to the liver have obscured well designed attempts to delineate and characterize agent-specific effects on the liver much less the signaling pathways involved; (2) There is accumulating evidence, which seems to indicate that buildup of mutations in hepatic alcohol metabolizing enzymes (alcohol dehydrogenase, aldehyde dehydrogenase, CYP2E1) and genetic alterations induced by alcohol in hepatic cells[13,14] may have further obscured attempts to elucidate the signaling pathways in ALD and fibro-hepatocarcinogenesis; and (3) Perhaps the most major difficulty is the bidirectional origin of ALD and fibro-hepatocarcinogenesis (gut to liver or liver to gut) and the dysregulation of key homeostatic functions (inflammation, immune regulation and regulation of fibrogenic signals). Notably, hepatic metabolism of alcohol as well as effect of alcohol on the gut generates many toxic chemical species with different mechanisms of hepatic toxicity which makes it difficult to distinctly identify their individual effects and signaling pathways involved.

This review takes a close look at current perspectives and scientific investigations on the effect of alcohol and its metabolic derivatives on hepatic parenchymal and non-parenchymal cells, bidirectional origin of ALD as well as the subtle conspiracy at the molecular level involving inflammatory, immune and fibrogenic signaling pathways underpinning ALD and fibro-hepatocarcinogenesis. Specifically, we put into perspective the complex and integrative roles of TGF-β (a key fibrogenic cytokine), Smad proteins, and

MAPK signaling pathways which pathologically suffer complicity in ALD and fibro-hepatocarcinogenesis mainly due to up-regulation of PAI-1 gene (a key downstream target gene of dysregulated TGF-β/Smad signaling in fibro-hepatocarcinogenesis) as well as recruitment of inflammatory and immune signaling pathways to promote ALD and fibro-hepatocarcinogenesis. Of note, the pathological role of PAI-1 in liver fibrosis, cirrhosis and cancer in general has been reported[15]. And these pathological roles of PAI-1 may be linked to dysregulated TGF-β/Smad and MAPK pathways.

Transforming growth factor beta is a prototype of a superfamily of multi-functional cytokines including bone morphogenetic protein (BMPs), activin, inhibin, growth and differentiation factors, nodal, and anti-Mullerian hormone[16]. The TGF-β_1 subtype has been extensively studied, mainly due to its physiological and pathological roles in the regulation of metazoan development, differentiation and homeostasis. It is in the light of these that the TGF-β class of cytokines is seen as a necessary evil in metazoan biology. In fact, TGF-β signaling pathway plays an important role during embryonic development, normal physiological processes and disease states by regulating several cellular processes, including cell growth and differentiation, cell migration, apoptosis, extracellular matrix formation[16] and inhibition of cell proliferation in the early stages of carcinogenesis mainly by blocking uncontrolled proliferation of epithelial, endothelial and hematopoietic cells[17]. However, genetic and epigenetic alterations of the TGF-β ligand, TGF-β-specific membrane receptors (TβR Ⅰ, TβR Ⅱ and TβR Ⅲ), and mediators (Smad proteins) may switch its tumor suppressor effects into tumor promotion. The susceptibility of TGF-β to loss of function mutations in various cancers has been reported[18]. For example, loss or gain of function mutations in TβR Ⅰ[19] TβR Ⅱ[20-22], Smad2[23,24], Smad3[25] and Smad4[23,26] have all been implicated in various human cancers. Therefore, it is not surprising that dysregulated TGF-β signaling pathway suffer complicity in almost all known human cancers[27-30]. It is maintained that genetic and epigenetic factors conspire to mastermind switching of TGF-β function by rendering tumor cells resistant or unresponsive to TGF-β-mediated growth arrest, and other homeostatic functions. TGF-β has been branded as the key factor regulating the acquisition of all the phenotypic hallmarks of cancer (cell proliferation, induction of epithelial to mesenchymal transition (EMT), induction of tissue invasion and migration, induction of tumor angiogenesis, inhibition of immune surveillance, induction of cancer cell survival, cancer cell immortality and resistance to TGF-β-mediated cytostasis)[27,30]. Recent evidence shows that mitogen activated protein kinase (MAPK) pathway regulate linker-dependent phosphorylation of receptor mediated Smads (Smad2 and Smad3) to promote pathological roles of dysregulated TGF-β/Smad signaling in liver fibrosis and hepatocellular carcinoma (HCC)[31,32]. The question arises as to how these signaling pathways act in synchrony to modulate alcohol-dependent activation of the hepatic cells to promote ALD and fibro-hepatocarcinogenesis from the perspective of the gut and the liver. Does chronic alcohol exposure alter TGF-β/Smad and MAPK signaling pathways? If it does, how and which component of the TGF-β/Smad signaling mediators is/are altered and how? Finally, how do deliberations on these questions inform us of future research directions and therapeutic strategies against ALD and fibro-hepatocarcinogenesis? The above questions are the preoccupation of the present review.

HEPATIC ALCOHOL METABOLISM

The liver metabolizes alcohol by employing two mechanisms, either through cytosol degradation by alcohol dehydrogenase to acetaldehyde (AA), then to acetic acid by aldehyde dehydrogenase in the mitochondria or *via* the cytochrome P450 (CYP) isoenzyme system where CYP2E1 actively metabolizes alcohol in cases of heavy alcohol ingestion[33-35]. Efficient functioning of these two hepatic alcohol metabolic processes ensure that toxic metabolites of alcohol, mainly AA (a hepatotoxin as well as a neurotoxin), MDA (a hepatotoxin) and some other unstable derivatives of the metabolites including CYP2E1-generated free radicals, protein adducts of AA and MDA, are rendered inactive or cleared from the system long before they cause any cellular damage. Indeed, buildup of AA and MDA, an inevitable phenomenon in chronic alcohol intake, is implicated for most of the toxic effects associated with chronic alcohol use[34].

Interestingly, it was reported that CYP2E1 activity may be induced about two to tenfold after chronic alcohol exposure and the underlying mechanism was linked to oxidative stress[36]. It was also reported that CYP2E1-dependent alcohol metabolism causes oxidative stress through increased output of reactive oxygen species (ROS)[37-39], which has already been implicated in lipid peroxidation and liver injury[40].

It must be noted that both cytosolic and mitochondrial alcohol metabolic pathways reduce NAD^+ to NADH (addition of a hydrogen atom to NAD^+ to convert it to NADH), however, impairment of any of the two metabolic pathways as a result of chronic alcohol intake may lead to a high NADH/NAD^+ ratio which by extension affects cytosolic and mitochondrial metabolism of carbohydrate and lipid substrates leading to impaired gluconeogenesis[4]. It was reported that alcohol exposure induces fatty liver disease by increasing NADH/NAD^+ ratio[41]. It remains to be established whether alcohol-induced NADH/NAD^+ turnover underlies reprogramming and switching energy metabolism of pre-neoplastic hepatic cells from efficient mitochondria oxidative phosphorylation to that of inefficient but protective aerobic glycolysis (so-called Warburg effect). The net effect is that there is

diminished substrate flow through the Kreb's cycle, giving rise to diversion of acetyl CoA to fatty acid synthesis and this possibly underlies NADH-induced inhibition of mitochondria fatty acid β-oxidation and elevated fatty acid synthesis leading to the onset of alcoholic liver disease[42-44].

Currently, it has been proposed that the pathogenesis of a healthy liver to one of alcohol-induced liver damage may involve a two-hit progression with steatosis being considered as the "first hit", followed by cellular insults such as oxidative stress, lipid peroxidation, direct lipid toxicity, mitochondrial dysfunction and/or infection to cause hepatic inflammation leading to alcoholic steatohepatitis[4-6]. As useful as this current "two hit" proposal may be, it remains to be clarified whether the pathological sequence of ALD leading to fibro-hepatocarcinogenesis lend itself to any particular set pattern, in view of the fact that diverse toxic agents of non-alcoholic origin may also influence ALD progression. The effect of co-morbidity factors such as hepatitis B and C infections has been shown to increase the progression of ALD. However, it is still difficult to clarify the question of which toxic agent first initiates liver damage and which toxic agent takes over at what cellular time scale and how? Is it alcohol or the co-morbidity factors that first initiate liver damage? It appears that alcohol-induced liver damage leading to ALD and fibro-hepatocarcinogenesis may not follow any specific temporal sequence, in view of the presence of other non-alcohol toxic agents. It is possible that the underlying non-alcohol liver-specific toxic agents may be the determinants of the temporal sequence of alcohol-induced liver disease.

Alcohol-induced liver damage displays bidirectional origin in view of the significant nauseating role of lipopolysaccharide (LPS) derived from progressive alteration of the gut microenvironment by chronic alcohol intake.

ALCOHOL AND ALTERATION OF GUT MICROBIOME

The invention of the microscope[45] provided the impetus to uncover the co-existence of micro-organisms and humans[46]. It is now a common knowledge that the human gut, (a prominent barrier organ) harbors a metagenomic community of some 10^{14} micro-organisms[47] mainly dominated by bacteria. The gut microbiome does play many regulatory functions spanning regular modulation of the innate and adaptive immune systems[48], synthesis and release of nutrients, vitamins, and preservation of the structural and functional integrity of the gut wall[46]. For instance, in the course of evolution of the gut microbiome, the diverse gut microorganisms have progressively managed to adapt as commensals, producing nutrients, such as vitamins of the B and K subclasses[49], and short-chain fatty acids (SCFAs). About 60%-90% of SCFAs in the gut lumen are absorbed by enterocytes to regulate energy supply, control gut pH, and resist pathogenic growth[50] probably *via* inflammasome[51]. The gut microbiome also plays a role in bile acid regulation[52,53], exchange of phenolic and aromatic acids[54], cholines, fatty acids and phospholipids[55,56]. Liver-specific biosynthesis of primary bile acids are reported to be dehydroxylated by some of these gut microbiome giving rise to secondary bile acids, which may be absorbed by the enterocytes to promote lipid absorption and energy homeostasis[52,53]. In view of the above, the importance of the gut microbiome in anti-oxidant, inflammatory, immune and energy homeostasis cannot be underestimated and therefore it represents a crucial determinant of the body's susceptibility to irritants including alcohol and its metabolic derivatives.

It is not surprising that alterations in the number and species diversity of the gut microbiome culminating from host-behaviors including but not limited to chronic alcohol intake derail the essential benefits of the gut microbiome[57] and may provide avenue for the onset of various inflammatory diseases of the gastrointestinal system and its accessory organs, of which the liver is the most affected. From hindsight, change in gut microbial diversity has long been implicated in Crohn's disease (CD)[46], ulcerative colitis (UC)[46] and irritable bowel disease (IBD)[46,58]. The case is not different with chronic alcohol exposure and the possible increase in Gram negative/Gram positive bacteria ratio (Figure 1). Nakayama *et al*[59] have shown that increased translocation of *Streptococcus suis* and its degraded products across the gut wall secondary to alcohol exposure correlated with progression of ALD. Accumulating evidence show that chronic alcohol intake may switch the aforementioned essential regulatory functions of the gut microbiome into a rather deleterious one. For example, alcohol-induced gut dysbiosis increases endotoxin turnover[60,61], particularly LPS[6,62], which leads to increased leakage of endotoxins into portal circulation and chronic stimulation of the liver. By diverse mechanisms, alcohol and its metabolic derivatives have been implicated in dysbiosis of the gut mucosal layer[63-66].

Lipopolysaccharides (LPS) are breakdown products of bacterial cell walls, specifically pathogenic Gram negative bacteria strains[67] and it is reported that it can activate hepatic cells[6,67] and initiate overt inflammatory responses *via* TNF-α mediation[68,69]. Under normal physiological conditions, release of LPS from breakdown of pathogenic Gram negative bacteria into portal circulation is rendered harmless by endothelial cells lining blood vessels, sinusoidal endothelial cells (SECs) of the liver as well as liver-resident macrophages (KCs) or its cellular concentration is reduced to levels well below physiological concentrations insufficient to elicit any inflammatory response. But perturbations of the

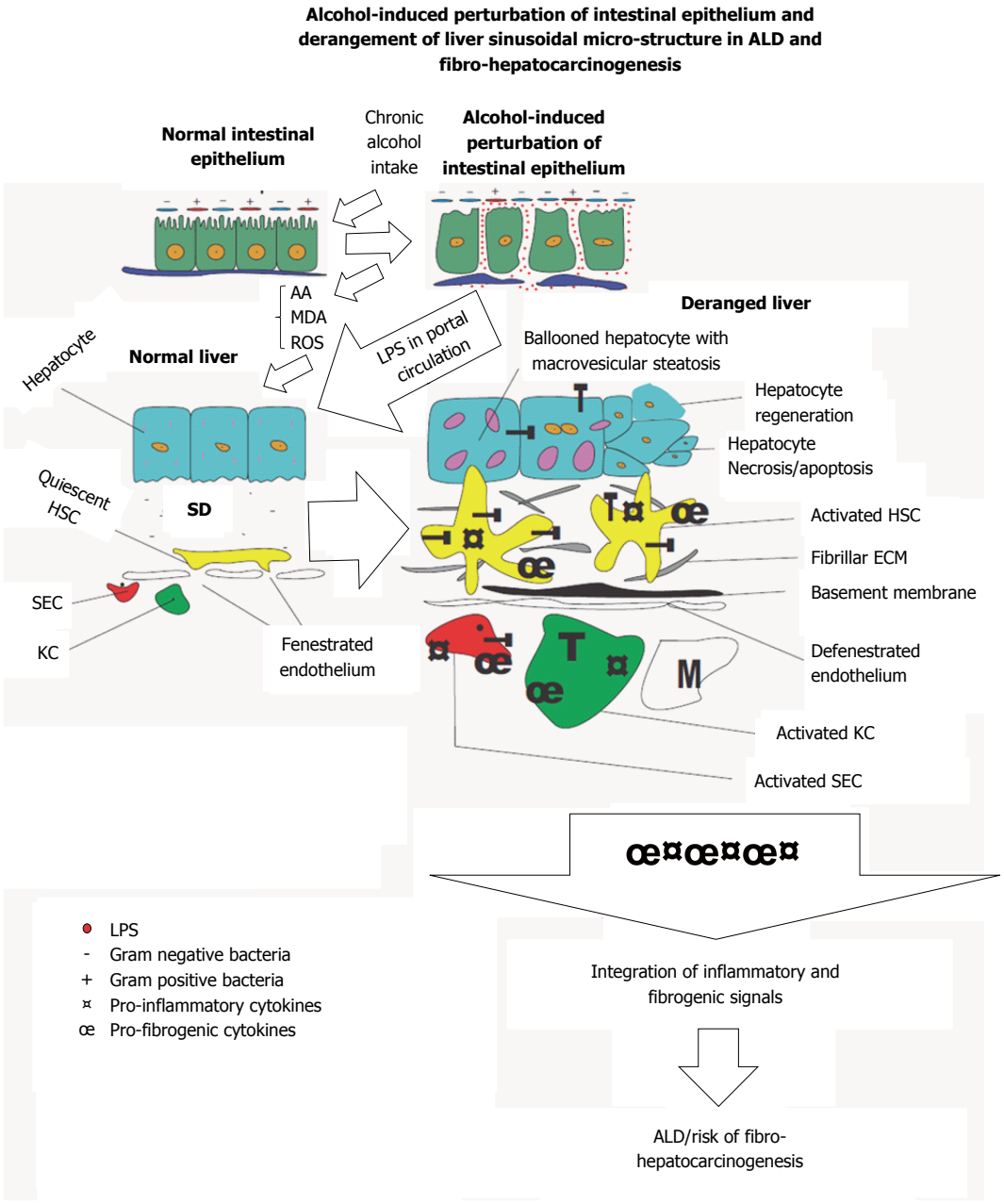

Figure 1 An illustration of the bidirectional origin of alcoholic liver disease and fibro-hepatocarcinogenesis within the gut-liver axis. Chronic alcohol use induces derangement of the gut epithelium, increases Gram-/Gram+ bacteria ratio, increases endotoxin turnover, increases permeability of gut epithelium to endotoxins including lipopolysaccharide (LPS). Subsequently leakage of LPS into portal circulation gain access to liver to initiate activation of hepatic cells. LPS-dependent activation of hepatic cells is further augmented by metabolic derivatives of alcohol to promote alcoholic liver disease (ALD) and fibro-hepatocarcinogenesis. T: Toll-like receptor 4; HSC: Hepatic stellate cell; KC: Kupffer cell; SD: Space of disse; SEC: Sinusoidal endothelial cell; M: Monocyte.

gut wall as a result of chronic alcohol intake (Figure 1), increases gut permeability to LPS derived from degraded bacterial cells[62] and this certainly leads to leakage of high concentrations of LPS into portal circulation. The high levels of LPS in portal circulation overwhelms the regulatory capacity of SECs and endothelial cells leading to chronic liver injury[41]. Continual exposure of the liver to gut-derived LPS serves as an inflammatory nosae, first by disrupting the balance between inflammatory and anti-inflammatory homeostatic regulation. This balance is shifted to favor heightened or sustained inflammatory response. To sustain the exaggerated inflammatory response, LPS first activates hepatic parenchymal cells, precisely SECs KCs and hepatic stellate cells (HSCs) leading to re-programming of their core functions. It is not surprising that a correlation was reported between increased intestinal LPS permeability and alcoholic hepatitis[60,61]. Similarly, LPS derived from alcohol-induced increase in gut permeability was shown in alcoholics to correlate with the pattern and the amount of alcohol consumed[70,71] while high levels of LPS were detected in the sera and livers of patients with alcohol-induced liver disease[62]. The hepatotoxic effect of pathogen associated molecular patterns (PAMPs), *e.g.*, LPS[72] has been shown to be mediated through toll-like receptors (TLRs)[73]. LPS is specifically reported

to be the ligand for TLR4 subtype[67]. Importantly, TLR4 as well as other TLR subtypes have been shown to be expressed on KCs, HSCs and hepatocytes under inflammatory conditions[9,74]. TLRs are crucial in the regulation of innate immune responses, sensing of damage associated molecular patterns (DAMPs) as well as PAMPs, of which LPS is an integral component. Also, it was reported that LPS/TLR4 signaling involves LPS-binding protein (LBP), CD14 and MD-2[75,76]. LBP facilitates the transfer of LPS from the outer membrane of bacterial cells to CD14, which in turn ensures the formation of TLR4-MD-2[77] to trigger LPS/TLR4 signaling, but downstream of this TLR4-mediated LPS-induced liver inflammation is myeloid differentiation factor protein 88 (MyD88). LPS-induced release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-12), chemokines (INF-γ, MCP-1)[78] as well as cell adhesion molecules (ICAM-1, VCAM-1) *via* activation of NF-κB and IκB in injured hepatic cells was MyD88-dependent[78]. LPS-induced activation of the MAPK pathway leading to the expression of activator protein (AP)-1 was also reported to be MyD88-dependent[79-81].

In short, chronic alcohol intake may generate an inflammatory arsenal comprising LPS, ROS, AA and MDA together with their respective protein adducts, which launches continuous attack on hepatic cells (Figure 1). The pathological manifestation of these acohol-mediated attacks on the liver may depend in part on the synergistic interaction between alcohol and non-alcohol co-morbidity factors. Consequently, this sets the stage for the onset of liver fibrosis and its progression to cirrhosis, thus increasing the risk of fibro-hepatocarcinogenesis. To appreciate how the alcohol generated inflammatory arsenal attack the hepatic cells, we take a cursory look at each of the hepatic cells in the light of their normal structure and function *vs* their alteration by alcohol and its metabolic derivatives.

ALCOHOL, METABOLIC DERIVATIVES OF ALCOHOL AND ACTIVATION OF HEPATIC CELLS

Sinusoidal endothelial cells

Sinusoidal endothelial cells (SECs) are hepatic non-parenchymal cells characterized by flattened and highly fenestrated features[6]. SECs have no basement membrane and their microstructure endows them with the ability to selectively filter blood components from portal circulation into the space of Disse, for subsequent presentation to the hepatocytes and lipid storage cells[6].

Additionally, SECs are endowed with scavenger receptors (SRs) and higher permeability properties, and these factors make it possible for SECs to engage in phagocytosis, clearing blood of harmful toxins and ensuring bidirectional exchange of substances between the hepatic parenchyma cells and portal blood. The SECs, by logic provides the first line of defense for the liver, essentially scavenging and clearing potential harmful products from attacking the liver. Many immunological functions, including but not limited to the following, removal of small molecules (< 200 nm) from the blood using innate immune mechanisms such as scavenger and mannose receptors[82,83], expressions of MHC class Ⅱ and co-stimulatory molecules (CD54 and CD106), and antigen processing, presentation, and leukocyte recruitment[83], have been attributed to SECs.

The afore-mentioned homeostatic functions of SECs in the liver of an alcoholic become derailed due to continuous and sustained activation of SECs by gut-derived LPS leakage into portal circulation. For example, exposure of SECs to LPS was shown to have induced basement membrane formation[84], and this observation was said to have preceded fibrogenesis. Some results from *in vitro* studies have also shown that SECs can reverse activated HSCs back to their quiescent state, but SECs loose this property following capillarization due to LPS activation[85]. Serious is the fact that continuous LPS-activation of SECs induces decreased responsiveness of SECs to LPS and also diminishes SEC-dependent scavenger functions. Eventually, the liver may be exposed to potentially damaging insults including metabolic derivatives of alcohol.

Accordingly, AA and MDA were shown to have formed protein adducts, which in turn stimulated SECs to produce more fibrogenic cytokines[6]. A clear attestation to this observation was demonstrated by fibronectin. Fibronectin was shown to be overexpressed following alcohol-induced liver damage and it was implicated in the activation of HSCs leading to liver fibrosis[6]. MDA-derived protein adducts were reported to have increased the expression of soluble fibronectin, cellular fibronectin and EⅢA fibronectin variant (the variant form of fibronectin, most implicated in HSC activation)[86,87]. Pro-inflammatory cytokines including TNF-α, MCP-1, and MIP-2 were similarly shown to have increased following treatment of isolated SECs with MDA-derived protein adducts[87,88]. Also, AA/MDA modified proteins were reported to have induced SECs to release both pro-inflammatory and pro-fibrogenic signals[88], while LPS was similarly reported to induce AA/MDA modified protein adduct-dependent release of chemokines and cytokines by SECs[89]. Evidently, LPS, AA, MDA, and their protein adducts act in concert to potently induce apoptosis of SECs which leads to weakening of SEC-dependent defense mechanisms of the liver, further, compounding an already compromised liver. LPS-activated SECs may in turn trans-activate KCs and HSCs in addition to their direct stimulation by LPS.

Kupffer cells

As part of the reticuloendothelial system are cells called Kupffer cells (KCs). Kupffer cells are monocyte-

derived cells resident in the liver as specialized hepatic macrophages[6,90]. These cells were first observed by Karl Wilherm von Kupffer in 1876[91] to whom is credited the name KCs, but it was not until 1898 that KCs were correctly identified as macrophages[92]. Their origin can be traced to the bone marrow, where pro-monocytes and monoblasts cells differentiate into monocytes, which then enter circulation and finally transform into KCs[93].

Functionally, KCs form a major part of the reticuloendothelial system within the liver sinusoidal compartment, phagocytizing senescence red blood cells as well as phagogenic presentations. As a result, they are widely scattered within the sinusoids. In support of the phagocytizing capacity of KCs, Helmy *et al*[94] have reported a receptor of the immunoglobulin family (CRIg), and they further showed that CRIg null mice could not clear complement system-coated pathogens, and that CRIg is well conserved in mice and humans, emphasizing the relevance of the CRIg as a component of the innate immune system and that of the role of KCs in the innate and complement systems.

In normal physiological state, KCs perform their immuno-regulatory functions without overt release of pro-inflammatory cytokines and chemokines; however, alcohol and its associated metabolic derivatives have the potential to reprogram KCs through repeated or continuous activation. This undue activation of KCs renders them more pro-inflammatory and pro-fibrogenic due to KC-dependent release of inflammatory and fibrogenic cytokines. For instance, it was reported that under stress conditions, KCs and other hepatic cells release cytokines (IL-1, IL-6, IL-8, TNF-) and chemokines (MIP-2, IP-109, KC/GRO, MIP-1α, and RANTES)[90,95]. It is the unregulated release of these inflammatory and fibrogenic cytokines that induces liver injury. It was further shown that each of the pro-inflammatory cytokines and chemokines could directly cause liver injury by targeting hepatic cells or indirectly through chemo-attraction of immune cells including neutrophils and lymphocytes[95]. Also, it was reported that the expression of adhesion molecules changes during LPS/alcohol-dependent liver injuries. Notably the enhanced expression of PECAM-1 and down-regulation of ICAM-1 characteristic of normal liver were reported to be reversed by TGF-β under inflammatory conditions[96].

Chronic exposure of the liver to alcohol succeeds in changing the sensitivity of KCs to LPS stimulation[97-99]. In a study to clarify alcohol-induced sensitization of KCs to LPS, Watanabe *et al*[100] have suggested that it could be due to the effect of alcohol on calcium channels, which is indispensable for TNF-α release. Exposure of KCs to alcohol does not only increase sensitivity of KCs to metabolites of alcohol but also increases intracellular calcium channels in KCs. It was shown that KCs exposed to alcohol for two hours lacked elevated intracellular calcium, but prolongation of alcohol exposure time to 24 h showed increased intracellular calcium, which manifested as TNF-α production and expression of LPS binding receptor (CD14), and this perhaps explains the increased sensitivity of KCs to LPS. Chronic alcohol exposure was also shown to have increased the expression of α2A-adrenoceptors in activated KCs and it was linked to the release of TNF-α and TNF-α-induced liver injury[101]. LPS was shown to augment AA/MDA protein adduct-mediated release of pro-inflammatory and pro-fibrogenic cytokines and chemokines by KCs[89]. LPS-induced acute and chronic liver injury was linked to activation of KC[102,103].

The activation of KCs is not limited to only LPS-derived from gut microbiome, and proteins modified by AA and MDA may also activate KCs leading to overt inflammatory and immunological responses injurious to the liver (Figure 2). Many alcohol modified protein adducts have been widely implicated in alcoholic liver disease[89,104,105]. Alcohol and its metabolic derivatives have also been implicated in KC/prostaglandin E2-induced liver injury[106] and this is in part attributed to endotoxin-dependent release of nitric oxide (NO) in KCs[107]. It appears that, chronic alcohol exposure in addition to AA and MDA dependent liver injury may also induce increase in gut-derived LPS, increase in LPS leakage into portal blood, increase in blood concentration of LPS, increase in LPS binding receptor expression and increased sensitivity of KCs to LPS and these may collectively sustain liver injury.

LPS activation of hepatic cells shows a snowballing effect, in that apart from LPS directly activating each hepatic cell, each activated hepatic cell may in turn influence the activation of its neighboring hepatic cells in a manner akin to paracrine or hormone-like cell to cell communication. For example, LPS-induced activation of SECs leads to activation of KCs *via* release of pro-inflammatory and pro-fibrogenic factors, similar activation of KCs also in turn activate quiescent HSCs through the release of TNF-α and TGF-β_1[6]. Typically, LPS-activated KCs were shown to have activated HSCs *in vitro* leading to HSC proliferation and increased ECM production[108]. Next, we look at how alcohol and its metabolic derivatives act in concert to activate HSCs to usher in liver fibrosis.

Hepatic stellate cells

Hepatic stellate cells (HSCs) are non-parenchymal hepatic cells located within the space of Disse between endothelial cells and hepatocytes[109,110]. HSCs can exist in two distinct forms depending on whether they are activated by an external inflammatory nosae or otherwise. In their normal inactivated state, also known as quiescent stellate (Ito) cells, they function normally by storing vitamin A, and may play modulatory roles during inflammation by expressing ICAM-1 and VCAM-1. It has been shown that HSC activation (tran-differentiation of quiescent vitamin

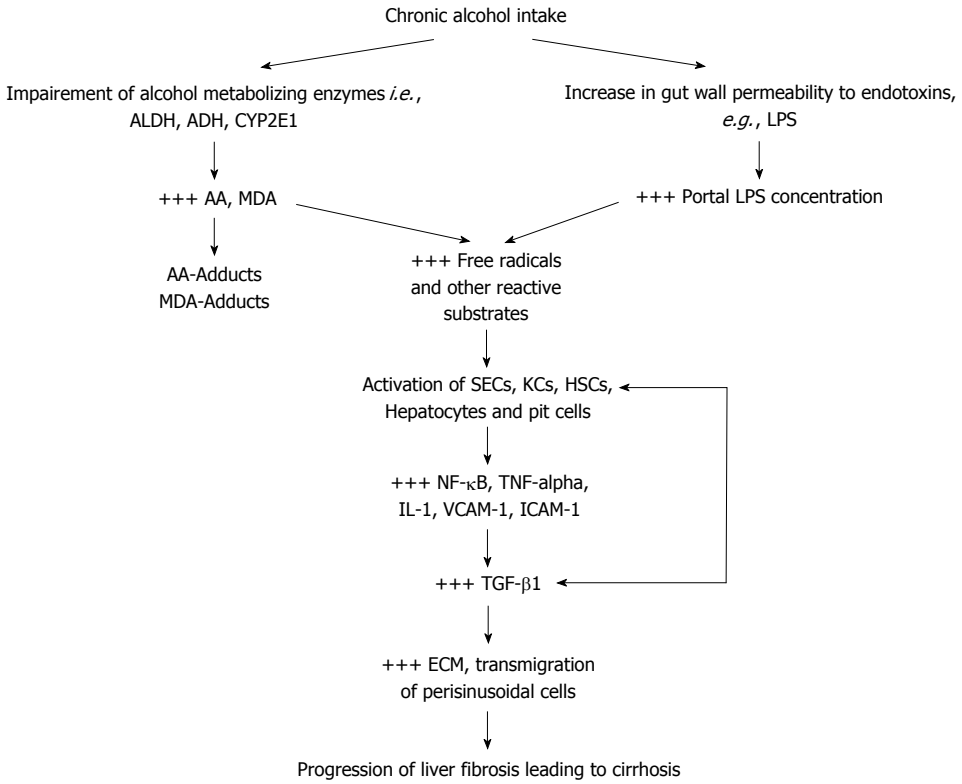

Figure 2 A schematic illustration of the two-way attack of hepatic cells in alcoholic liver disease and fibro-hepatocarcinogenesis. By multiple mechanisms alcohol metabolizing enzymes and gut-derived LPS induce production of free radicals which stimulate the release of pro-inflammatory and pro-fibrogenic cytokines. Free radical-dependent activation of hepatic cells leads to the release of pro-inflammatory transcription factor (NF-κB) and inflammatory cytokines (TNF-α, IL-1β) which provide the signal for injurious overt hepatic inflammatory response. Secondary to the injurious hepatic inflammatory response, is the activation of hepatic cells (mainly HSCs and KCs) to further release pro-fibrogenic factors, mainly the key fibrogenic cytokine (TGF-β) which mediates a high ECM turnover (increased fibrogenesis : fibrinolysis ratio) in HSCs and the space of Disse, and trans-migration of hepatic non-parenchymal cells. These pathological events initiate liver fibrosis and cirrhosis leading to fibro-hepatocarcinogenesis. AA: Acetaldehyde; ADH: Aldehyde dehydrogenase; ALDH: Alcohol dehydrogenase; ECM: Extracellular matrix; HSCs: Hepatic stellate cells; IL-1β: Interleukin-1 beta; KCs: Kupffer cells; LPS: Lipopolysaccharide; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa B; SECs: Sinusoidal endothelial cells; TGF-β: Transforming growth factor beta; TNF-α: Tumor necrosis factor alpha; VCAM: Vascular cell adhesion molecule; +++: Up-regulated expression or overproduction.

A-storing cells to proliferative myofibroblast cells) by hepatotoxins plays a crucial role in liver fibrogenesis. To substantiate this claim, it was shown that inactivation of HSCs attenuates liver fibrosis[111]. Activated HSCs, mostly release excess TGF-β1 which has been shown to down-regulate ICAM-1 and VCAM-1 while increasing N-CAM expression in HSCs. In addition, HSCs in their activated states may express TNF-α, which reduces N-CAM-coding mRNAs and also induces ICAM-I-and-VCAM-1 specific transcripts by many folds[90]. Under inflammatory conditions, HSCs trans-differentiate into myofibroblast and concomitantly increase ECM production. There is dysregulation of ECM metabolism which promotes liver fibrosis. This underlies the initiation of fibrogenesis and the complicity of HSCs in liver fibrosis[112]. Coherent with this, Knittel *et al*[113] have intimated the importance of HSCs during hepatic injury through the recruitment and migration of mononuclear cells in the peri-sinusoidal space. This perhaps sets the stage for the secretion of TGF-β and TNF-α and their pathological role in liver fibrosis[114,115] and several other cytokines and chemokines including MCP-1, RANTE-1, and IL-8[116-118].

The effect of alcohol and its metabolic derivatives on HSC activation and their roles in ALD progression have been extensively investigated. For example, alcohol-induced activation of HSCs was linked to the release of TGF-β1, matrix proteins, and initiation of fibrogenic response[112,119]. Karaa *et al*[120] have also shown that alcohol exposure to mice produced HSC activation in addition to increased hepatic collagen output and neutrophil infiltration.

Evidence implicating alcohol-specific metabolites in liver injury was clearly demonstrated by using 4-methylpyrazole (4-MP), an inhibitor of alcohol metabolism. It was shown that sustained alcohol exposure to precision cut liver slices (PCLS) produced increased levels of IL-6, depletion of GSH stores, increased lipid peroxidation, increased expression of smooth muscle actin (α-SMA) and increased deposition of collagen in liver sinusoidal space[6]. But all these aforementioned alcohol exposure-specific phenotypic hallmarks were reversed by 4-MP treatment[6,121], emphasizing the involvement of alcohol and its metabolic derivatives in liver fibrosis.

Furthermore, LPS was reported to exert direct

effect on HSCs[122] and also indirectly *via* LPS activated KCs[108,123]. LPS was shown to enhance the effect of alcohol and AA on HSC activation[124,125] and the expression of collagen 1 and IL-6[126], while AA alone induced activation and proliferation of HSCs[127,128] and expression of α-SMA[127,129].

At the mechanistic level, AA-induced activation of HSCs was linked partly to ERK1/2/PI3K pathway[130,131], JNK/α1-collagen pathway[132] and TGF-β1/TβRⅡ signaling[133,134]. Transcriptional elevation of TGF-β and its membrane receptors (TβRⅠ and TβRⅡ) have been considered as key hallmarks of activated HSC[135]. And this certainly increases the sensitivity of activated HSCs to TGF-β-mediated fibrogenic signals. Siegmund *et al*[9] have posited that the signal for ECM production by HSCs in liver-related diseases of all etiologies including alcohol emanates from TGF-β. Corroboratively, it was shown that TGF-β protein levels increased in both experimental and human liver fibrosis[136]. Thus, TGF-β is the key contributor of irregular ECM accumulation in liver sinusoidal space by forming basal membranes leading to defenestration of the liver sinusoids[137] as well as decreasing MMPs to further halt ECM degradation[138].

Hepatocytes

Hepatocytes are the main hepatic cells in the liver accounting for 80% of the cytoplasmic mass of the liver[90]. Characteristically, hepatocytes exhibit eosinophilic cytoplasm (a cytoplasm with abundant mitochondria) and basophilic stippling (abundance of endoplasmic reticulum and ribosomes)[90]. Within the liver, hepatocytes are organized into cell-thick plates separated by vascular channels[139,140]. When these structural and functional characteristics of hepatocytes are not disturbed or altered by any nosae, hepatocytes can attain an average life span of 5 months whiles retaining the capacity to regenerate[90].

Functionally, hepatocytes are involved in protein synthesis, protein storage, and transformation of carbohydrates, synthesis of cholesterol, bile salts, phospholipids, detoxification, modification and excretion of exogenous and endogenous substances[90]. Additionally, hepatocytes can biosynthesize hormones including insulin-like growth factor (IGF-1)[90,141], thrombopoietin[142], erythropoietin[143] and cytokines such as IL-8[144,145] needed for normal hepatic homeostatic processes.

In the event of liver injury due to alcohol and its metabolic derivatives, the degree of injury overwhelms the hepatocyte defense mechanisms, especially when the nosae is continuous and sustained (Figure 1). As a means of defense, hepatocytes may respond to acute phase inflammatory mediators such as IL-6 by releasing acute phase proteins including C-reactive protein (CRP)[146], serum amyloid A (SAA)[147] and also some intracellular defense proteins like heme-oxygenase-1 (HO-1)[148]. In a desperate attempt to fight back, hepatocytes employ various mechanisms including release of chemokines such as MIG[149], IP-10[149], cytokine-induced neutrophil chemo-attractant (KC), MIP-1, MIP-2, and MIP-3 which act in concert to recruit and activate pro-inflammatory cells (mononuclear phagocytes) and KCs respectively. But because the inbuilt intracellular hepatocyte defense mechanisms *i.e.,* CRP, SAA and HO-1, and the anti-oxidant system have already been weakened, by the continuous exposure of the damaging nosae, the hepatocyte defense response leads to mass hepatocyte apoptosis at a rate that further compromise the structural and functional integrity of the liver as a whole. Hepatocyte cytosol and microsomal compartments are the initial sites for alcohol metabolism before mitochondrial-dependent breakdown. The number and integrity of hepatocytes are rate-limiting factors in alcohol metabolism. Therefore, increased apoptosis of hepatocytes leads to poor alcohol metabolism which further increases the buildup of alcohol metabolites (AA and MDA), meanwhile KCs, SECs, and NKs which could clear the debris and the resulting buildup of toxins have been disabled by LPS, AA, and MDA. This situation exposes the liver to potentially damaging nosae which may set the stage for the initiation of cirrhosis leading to HCC.

Pit cells

Pit cells, lymphocyte-derived non-parenchymal cells, form about 1% of the non-parenchymal cell mass[150]. Pit cells are liver representatives of natural killer cells (NKs) in other organs. Pit cells are suspected to have originated from the bone marrow transported by blood to finally settle in the liver, where they transmogrify into their current state by lowering their density and increasing their granular content. The very existence of the pit cells are dependent on KCs[151], which suggest that whatever that happens to KCs will in turn affect the fate of pit cells.

Functionally, pit cells biosynthesize interferon gamma (IFN-γ) in response to damaging inflammatory nosae, but they can also partake in the destruction of virus-infected malignant cells. Pit cells are versatile migratory cells and they are normally activated by interleukin-2[90]. Alcohol, AA, MDA, and LPS may directly damage pit cells through continuous activation or indirectly by activated KCs and HSCs, leading to functional impairment and consequences thereof on the liver, in view of the fact that pit cells *via* the perforin/granzyme-dependent mechanism are indispensable in the removal and apoptosis of splenic/blood-NK-resistant tumor cells[152]. Importantly, TGF-β-induced repression of NKs, a phenomenon characteristic of chronic alcohol consumption[153], has been linked to failure of NK cell-mediated apoptosis of HSCs[154,155].

Evidently, chronic alcohol intake affects the structural and functional capacity of all the hepatic

cells and this possibly may set the cellular stage for the actions of pro-inflammatory and pro-fibrogenic cytokines acting in concert to promote ALD leading to fibro-hepatocarcinogenesis. At the center of this complex cellular conspiracy against the liver are the TGF-β/Smad and MAPK pathways and their downstream target genes.

TGF-β: A VERSATILE SIGNALING MODULATOR WITH COMPLEX FUNCTIONALITY IN ALD AND FIBRO-HEPATOCARCINOGENESIS

To appreciate how alcohol and its metabolic derivatives alter TGF-β/Smad signaling to promote ALD and fibro-hepatocarcinogenesis, we should first take a panoramic view of TGF-β/Smad signaling and also the cell/context-specific functions of TGF-β which perhaps may explain its complex and integrative roles in ALD. TGF-β is considered to exert growth restraints on various cancer cells at the initial stages of fibro-hepatocarcinogenesis by mechanisms including cell cycle arrest at critical check points, induction of apoptosis and restoration of cellular structure[156-158], an observation which highlights TGF-β as a potent anti-tumor cytokine[31]. However, by a sudden twist of events, in a different cellular context such as in ALD, fibro-hepatocarcinogenesis as well as in other disease pathologies, it tends to promote disruption of cell adhesion, induces migration and invasion, and mediate immune suppression and angiogenesis, to become a crucial tumor promoter[158]. For example, in ALD it was reported that AA does not only increase the steady levels of TGF-β mRNA transcripts[159] but also promote activation of latent TGF-β and elevate the expression of TβR Ⅱ [134]. Furtheremore, it was shown that a decrease in TGF-β protein levels correlated with a decrease in AA-induced α2 collagen (I) gene[160].

Basically, TGF-β signaling involves two major signaling modes, canonical and non-canonical. The former is mediated by Smad proteins while the latter involves cross signaling between TGF-β and other cell signaling pathways implicated in cancer such as Wnt/β-catenin pathway[161,162], VEGF pathway[163,164], aberrant FGF/FGFR signaling pathways[165,166], MAPK pathway[167,168], PI3k/AKT/ mTOR pathway[169], HGF signaling[164], aberrant EGF/EGFR signaling pathway[170], and deregulation of IGF pathway[171]. To proceed, we highlight the Smad proteins and the MAPK pathway, which have so far been shown to work closely with TGF-β signaling in both cell and animal models of ALD (Table 1) to promote liver fibrosis and HCC[31].

Smad proteins

Genetic studies spanning more than a decade ago using *Caenorhabditis elegans* (a nematode), and *Drosophila melanogaster* (a fruitfly) led to the discovery of a group of genes, which were later named Smads from their original sources. Smads are the central mediators that carry signals from receptors of TGF-β, BMP, and activin cytokines to the nucleus[172]. Smads which are now identified as substrate transcriptional factors play integral functions in the intracellular signaling responses to TGF-β and its related signaling complex[173]. Basically, the Smad proteins have three forms, namely receptor mediated Smads (Smad 2 and Smad3 specific for TGF-β), inhibitory Smad (Smad7) and a common Smad (Smad4). It must be stated that mutations in these Smad types due to genetic and epigenetic causes such as alcohol exposure are linked to dysregulated TGF-β signaling in ALD and cancer in general. For example, Smad7 inhibits TGF-β/TβRI-dependent phosphorylation of Smad2 and Smad3[174-176] to abrogate dysregulated TGF-β/Smad signaling transduction in many disease pathologies including but not limited to ALD and fibro-hepatocarcinogenesis. But, alcohol and LPS were shown to down-regulate Smad7 expression to induce liver fibrosis in a Smad3-dependent fashion[120]. Similarly, a number of reports from cell and animal studies (Table 1) have implicated receptor-mediated Smad proteins (Smad2 and Smad3) in ALD as well as in HCC patients. Smad4 deletions have been mapped in HCC[18]. Smad2[23,24], Smad3[25] and Smad4[177] mutations have been detected in various cancer subtypes. But it remains to be elucidated how alcohol and its metabolic derivatives modulate the Smad proteins and the signaling pathways they mediate, particularly the upstream (TGF-β ligand, TβR Ⅰ, TβR Ⅱ, Smad2, Smad3, Smad4, Smad7) and downstream (Imp7/8, PAI-1) signaling mediators of TGF-β and how such findings may inform future research directions and precisive therapeutic strategies against ALD and fibro-hepatocarcinogenesis. It is worth suggesting that Smad2, Smad3, and Smad7 deletions or/and mutations should be mapped in ALD as well as in HCC to facilitate early screening, diagnosis and treatment of ALD and its related complications.

Importantly, it must be mentioned that the switch of TGF-β function from tumor suppression in early stages of cancer as well as in early stages of ALD to tumor promotion in late HCC reflects an imbalance between canonical and non-canonical TGF-β signaling and recruitment of other oncogenic signaling pathways (Figure 3). What actually causes this switch has so far remained elusive. Refreshingly, in HCC it was reported that binding of Gas6 ligand to Axl induces activation of Axl/14-3-3ζ to switch TGF-β signaling from tumor suppression to tumor promotion in a JNK/Smad3L-dependent fashion[178]. It was also mentioned that suppression of Axl succeeded in blocking oncogenic TGF-β signaling in HCC, and this has raised hopes about indirect inhibition of oncogenic TGF-β signaling using Axl-specific inhibitors. A number of Axl-specific inhibitors are under various stages of preclinical studies including SGI-7079, BGB324, DP3975 and NA80xl[179].

Table 1 Involvement of Transforming growth factor-β, Smad, plasminogen activator inhibitor-1 and mitogen activated protein kinase signaling pathways in alcoholic liver disease and fibro-hepatocarcinogenesis

Alcohol/ metabolic derivative	Mechanism/pathway	Cellular context	Ref.
Alcohol, LPS, SAMe	Inhibition of TGF-β/Smad signaling abrogates alcohol-induced liver injury alcohol and LPS induce liver fibrosis via activation of TGF-β signaling in a Smad3-dependent fashion and down-regulation of Smad7, but SAMe could abrogate it and also restore Smad7 expression	Cultured HSCs, male rats	[120,221]
AA	Up-regulation of Smad3 and Smad4, increase in nuclear translocation of Smad3/4 complex, decrease in Smad7 expression, all leading to enhanced expression of COL1A2 Increase in TGF-β1 secretion and up-regulated expression of TβRII in HSCs was linked to AA AA increased COLα1 expression in HSCs in a Smad3-dependent manner	Human and mouse HSCs	[159,219,222,223]
Alcohol	Alcohol-induced increase in endotoxemia linked to up-regulated protein expression of TGF-β1, IL-6, NF-κB, TNF-α, IκBα	Guinea pig liver	[224]
Alcohol, LPS	Alcohol potentiates LPS-induced pancreatic fibrosis *via* increased production of TGF-β1	Human pancreatic tissue sample, pancreatic acinar-like cells (AR42J)	[225]
Alcohol	Alcohol-induced translocation of *S. suis* across gut wall and also up-regulated TGF-β1 and COL 1 to promote liver disease	Alcoholics, mice, Caco-2 cells	[59]
Alcohol	While alcohol exposure impairs nuclear import of growth hormone-induced STAT5B and IL-6-induced STAT3, it had no effect on TGF-β1-induced nuclear import of Smad2/3 TGF-β1 mediates liver fibrosis in experimental rats in a Smad4-dependent fashion Alcohol-induces hepatic iron overload leading to liver damage *via* modulation of hepcidin through BMP6/Smad4 signaling pathway	Rat liver, adult male C57BL/6J mice	[11,184,226,227]
Alcohol	Alcohol modulates iron-induced liver injury *via* increased expression of TGF-β1, BMP2, phosphorylated Smad2	Mice	[228]
Alcohol	Alcohol-induced steatosis and liver injury in Smad7 null mice is enhanced by TGF-β1 signaling and TGF-β1-induced EMT in hepatocytes	Alb-Cre mice, Smad7 (loxP/loxP) mice	[229]
Alcohol	Alcohol exposure induces TGF-β1 release and activation of TGF-β1-induced down-regulation of alcohol dehydrogenase 1 (ADH1) mRNA transcripts in part through TGF-β/ALK5/Smad2/3 signaling	Mice	[230]
Alcohol, AA	Alcohol and AA-induced activation of TGF-β1, JNK and p38 signaling pathways were inhibited by butein	HSCs, HepG2 cells	[231]
Alcohol	TGF-β1 mediates alcohol-induced activation of HSCs *via* activation of p38/JNK MAPK pathway and overexpression of HSC markers including α-SMA, procollagen1, betulin and betulinic acid can reverse these pathways to restore liver integrity	Rat HSCs	[232]
Alcohol, LPS	LPS and CYP2E1-dependent oxidative stress synergistically activate p38/JNK pathway *via* LPS/TNF-α signaling pathway	Hepatic cells	[233]
Alcohol	Alcohol induces cytotoxicity *via* activation of p38, JNK and ERK MARK pathway, but COS reversed this by inhibiting the MAPK pathway and activation of Nrf2	Human L02 normal liver cells	[234]
Alcohol	Alcohol induces hepatotoxicity by activating p38/JNK MAPK pathway in addition to NF-κB, IL-6, TNF-α, but these effects were reversed by MA	Mice	[235]
Alcohol	Activation of JNK and ERK MAPK pathway mediates alcohol-induced oxidative stress, but HO-1-derived CO reversed these effects by activating p38 MAPK pathway just as CORM-2, which suppressed TNF-α and IL-6	Adult male Balb/c mice, primary rat hepatocytes	[236]
Alcohol	TLR2/4, p38/ERK MAPK pathway, IL-1β, TNF-α, COX-2 mediate alcohol-induced liver injury, but noni juice (NJ) effectively reverses alcohol-induced liver injury by modulating the above factors	Mice	[237]
Alcohol	Alcohol-induced hepatocyte apoptosis is mediated through activation of p38/JNK MAPK pathway and also involve Fas	Human liver adenocarcinoma (SK-Hep1) cells	[238]
LPS	LPS induces liver inflammation *via* multiple pathways including activation of p38 MAPK/Nrf2/HO-1, ICAM-1, VCAM-1, TNF-α	RAW264.7 cells, CLP-induced septic mice	[239]
Alcohol	ERK MAPK activation, increase in mRNA transcripts of egr-1 and PAI-1 are associated with alcohol-induced steatosis and hepatic necrosis	Rats	[240]
LPS	Activation of p38 MAPK pathway and COX-2 mediate LPS-induced liver injury, however, ES attenuates liver injury by modulating the above pathways	Sprague-Dawley (SD) rats	[241]
Alcohol	Activation of p38, JNK and ERK MAPK pathways and histone modification (acetylation, methylation and phosphorylation) mediates alcohol-induced hepatic cellular injury	Male SD rats	[242]
Alcohol	Alcohol enhances Fas-induced liver injury by activating p38/JNK MAPK pathway, increase caspase-3 and -8 and TNF-α	Mice	[243]

LPS	Alcohol induces CYP2E1 and LPS overproduction, and CYP2E1 sensitizes hepatocytes to LPS/TNF-α-dependent injury and this is mediated through activation of p38/JNK MAPK pathway		[244]
Alcohol	Inhibition of liver regeneration in partial hepatectomized rats is associated with alcohol-induced p38 activation and cyclin D1 expression	Male Wistar rats	[245]
Alcohol	Alcohol-induced inhibition of HO-1 is mediated through blockade of p38/ERK MAPK-dependent nuclear import of Nrf2, but quercetin can reverse this blockade to restore hepatoprotection against alcohol-induced oxidative stress	Human hepatocytes	[246]
Alcohol	Increased gastric mRNA transcripts was reported as a response to alcohol-dependent nosae on the gut wall, suggesting the protective role of PAI-1 in the gut	C57BL/6 mice, PAI-1-1-H/Kβ mice	[247]
Alcohol, LPS	Increase in PAI-1 correlated with progression of ALD PAI-1 was implicated in hepatic inflammation and fibrosis in a two-hit model of ALD involving alcohol and LPS Alcohol-induced increased in hepatic lipids was linked to up-regulation of PAI-1, but this was reversed by metformin	*In vitro* and *in vivo* models of ALD, mice	[199-201]

ALD: Alcoholic liver disease; HGF; Hepatocyte growth factor; ECM: Extracellular matrix; PAI: Plasminogen activator inhibitor; PI3K: Phosphatidylinositol 3-kinase; EGF: Epidermal growth factor; MAPK: Mitogen activated protein kinase; TGF-β: Transforming growth factor beta; Smad: Small mother against decapentaplegic; SAMe: S-adenosyl-L-methionine.

Canonical TGF-β signaling

The canonical TGF-β signaling is mediated primarily by Smad proteins (Smad2, Smad3, Smad4) *via* TGF-β-specific receptors (TβRⅠ, TβRⅡ and TβRⅢ). Trans-membrane TGF-β signaling begins with ligand binding of TβRⅢ, which then presents TGF-β to TβRⅡ[180], this is peculiar to TGF-β2 which only interacts with TβRⅢ before it becomes bound to TβRⅡ. However, the other TGF-β family members, precisely TGF-β_1 and TGF-β_3 readily bind to TβRⅡ without the need of binding to TβRⅢ; therefore they can transduce intracellular signals either in the presence or absence of TβRⅢ[17]. TGF-β activated TβRⅡ subsequently trans-phosphorylate TβRⅠ. Activated TβR-Ⅰ in turn trans-phosphorylate latent transcriptional factors (Smad2 and Smad3) at their C-terminal SXS motif[17]. The phosphorylated Smad2/3 undergoes a rapid conformational change which facilitates their oligomerization with a common Smad4. The formation of Smad2/3/4 complex enhances preferential nuclear relocation and accumulation of the complex[181-183]. The cellular responses to TGF-β are fine-tuned by continuous nucleocytoplasmic shuttling of Smad2/3, which permits continuous sensing and responds to changes in TGF-β receptor activity[184,185]. The nucleocytoplasmic shuttling of Smad2/3/4 complex is crucial for TGF-β signaling and this is aided by karyopherins, particularly Imp7/8. In the nucleus, the Smad2/3/4 complex interacts with transcriptional co-activators or repressors to determine the transcription of TGF-β target genes, such as PAI-1 gene, therefore deciding the fate of cells[186].

Though alcohol and its metabolic derivatives in many studies (Table 1) have been shown to stimulate increased expression of PAI-1, it remains to be explored how alcohol and its metabolic derivatives modulate Imp7/8 which facilitates Smad2/3/4 nucleocytoplasmic shuttling. It must be noted that TGF-β/Smad signaling mediated through C-terminal phosphorylation of Smad2/3 corresponds to all its tumor suppressor and cytostatic functions. The exact modulation of canonical TGF-β/Smad, particularly Smad2/3/4 and their regulation by alcohol and its metabolic derivatives need to be clarified, in the light of gut-dependent and liver-dependent alcohol-induced inflammatory and fibrogenic signals. It is currently held that ALD may begin from the gut[187-190]. A paradigm that does not only highlight the therapeutic potential of the gut[191] but also the prognostic and diagnostic value of the gut. The possible crosstalk between LPS/TLR4/TNF-α/TNF-αRI/MLCK in gut dysbiosis/inflammation and alcohol-induced liver inflammation/fibrogenesis driven by dysregulated TGF-β/Smad and the MAPK pathways remain veiled. Unveiling the link between the gut-liver axis in the light of TGF-β/Smad/MAPK and TLR4/TNF-αRI signaling will undoubtedly not only help to explain the switch of TGF-β signaling from tumor suppression to tumor promotion in ALD, but will also open a new therapeutic avenue to advance target specific therapies against ALD and fibro-hepatocarcinogenesis.

Non-canonical TGF-β signaling

Aside the canonical TGF-β signaling *via* TGF-β-specific membrane receptors (TβRⅡ and TβRⅠ) and latent Smad proteins (Smad2, Smad3 and Smad4), TGF-β also activates other signaling pathways quiet independently. Among the signaling pathways activated by TGF-β are the MAPK pathway[192-194], the growth and survival kinases (P13K, AKT/PKB, mTOR) and the small GTP-binding proteins (Ras, RhoA, Racl, and Cdc42)[195-197]. It is worth notice that oncogenic non-canonical TGF-β signaling in its full activation as is the case in ALD and fibro-hepatocarcinogenesis far outweigh the canonical tumor suppressor TGF-β/Smad signaling and could therefore explain the connivance between alcohol and its metabolic derivatives and the oncogenic non-canonical TGF-β signaling in ALD, increasing the risk of fibro-hepatocarcinogenesis. This is evident in the elevated expression of PAI-1 in some cell and animal models of ALD and HCC. Importantly, PAI-1 is a key target gene of TGF-β signaling and plays

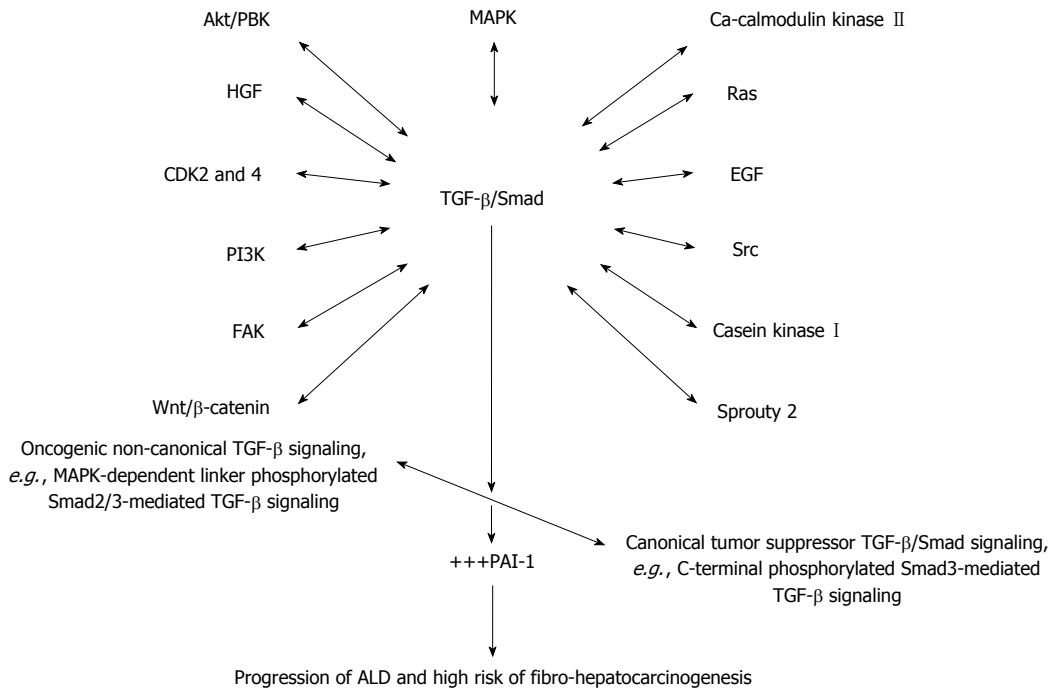

Figure 3 A proposed schematic illustration of the complex and integrative role of TGF-β/Smad signaling in alcoholic liver disease and alcohol-induced fibro-hepatocarcinogenesis. Alcohol and its metabolic derivatives induce the release and activation of TGF-β/Smad signaling through NF-κB/TNF-α mediation (Figure 1). The NF-κB/TNF-α-mediated activation of TGF-β/Smad signaling switches canonical tumor suppressor (C-terminal phosphorylated Smad3/Smad4-dependent TGF-β signaling) into oncogenic (MAPK-dependent linker phosphorylated Smad2/3-dependent TGF-β signaling) and also non-canonical TGF-β signaling involving cross-signaling with other signaling pathways implicated in hepatic malignancies. Key cross-signaling pathways which team up with TGF-β signaling includes but not limited to CDK 2 and 4, Ca-calmodulin kinase Ⅱ, EGF, HGF, PI3K/AKT, FAK, Src, Sprouty2, casein kinase Ⅰ, Wnt/β-catenin. This leads to imbalance between canonical and non-canonical TGF-β signaling. Increase in oncogenic TGF-β/Smad signaling leads to up-regulation of PAI-1 gene expression and PAI-1-mediated pathologies thereof. The key pathological effects of PAI-1 include dysregulated ECM regulation, cell proliferation and invasion, and dysregulated apoptosis and these underlie initiation and progression of alcohol-induced fibro-hepatocarcinogenesis. HGF: Hepatocyte growth factor; ECM: Extracellular matrix; PAI: Plasminogen activator inhibitor; PI3K: Phosphatidylinositol 3-kinase; EGF: Epidermal growth factor; MAPK: Mitogen activated protein kinase; TGF-β: Transforming growth factor beta; Smad: Small mother against decapentaplegic; +++: Up-regulated expression or overproduction.

crucial roles in all disease pathologies in which TGF-β is implicated.

Plasminogen activator inhibitor

Plasminogen activator inhibitor (*PAI*)-*1* gene is an important inhibitor of both tissue and urokinase type plasminogen activators; as a result it inhibits fibrin degradation *via* inactivation of plasminogen[6] and also mediates some inflammatory responses[198]. Many reports have implicated PAI-I in alcohol-induced liver damage[199-201]. PAI-I levels were shown to increase following acute and chronic alcohol exposure[200], and a decrease in PAI-1 expression perhaps through RNA interference significantly reduced alcohol-induced steatosis and lipid peroxidation[200].

Fibrinolysis has been shown to be a common feature in alcohol-induced liver disease; meanwhile PAI-1 gene is a crucial modulator of fibrinolysis through its inhibitory action on plasminogen activator[201]. The involvement of PAI-1 in alcohol-induced liver damage could be traced to the link between TNF-α and TGF-β/Smad signaling and could also explain the integrative role of TGF-β in the gut-liver axis in alcohol-induced fibro-hepatocarcinogenesis, since PAI-1 is a major target gene of TGF-β/Smad signaling pathway[31,32,202]. The complicity of TNF-α in alcohol-induced fatty liver disease, where it was purported to increase the expression of PAI-1,[6] emphasizes a more complex interaction between pro-inflammatory and fibrogenic factors in ALD. TNF-α mediates the pro-inflammatory signaling while TGF-β1 controls the fibrogenic signals. To confirm the TNF-α/PAI-1 link, alcohol-induced expression of PAI-1 in TNFR1 -/- mice was investigated and found inhibited[200], indicating that TNF-α might induce PAI-1 through the MAPK pathway[203]. But the crosstalk between the MAPK pathway and TGF-β/Smad signaling pathway is a common dysregulated signaling pathway implicated in many cancers, particularly MAPK-dependent linker phosphorylation of Smad2 and Smad3 leading to increased PAI-1 expression and occurrence of phenotypic hallmarks of fibro-hepatocarcinogenesis[32].

MAPK pathway

Recent developments in the understanding of TGF-β/Smad signaling has revealed non-Smad TGF-β signaling[204] and a growing indication of a crosstalk between MAPK pathway and Smad signaling downstream of TGF-β[205]. The MAPKs represent a large class of serine/threonine protein kinases crucial in the initial

responses to a diversity of extracellular signals involved in cell growth, cell differentiation and apoptosis[206] and activation of nuclear transcription factors by allowing nuclear sensing of extracellular signals[207]. They are grouped into three sub-classes: the extracellular signal-regulated kinases (ERK1, ERK2), the stress-activated protein (SAP) kinases, also known as c-jun N-terminal kinases (JNK1, JNK2, and JNK3), and the p38 MAPKs (α, β, γ, and δ)[193]. In several reports, TGF-β was shown to activate ERK, p38, and JNK in different cell types[208,209]. As a result, TGF-β signaling can be regulated *via* linker-dependent phosphorylation of Smad2/3. A number of protein kinases including ERK1/2[193], JNK[210] and p38[211] activated by TGF-β in turn phosphorylate Smad2/3 at the linker region. It is a common knowledge that linker-dependent phosphorylation of Smad2/3 in part switches the TGF-β/Smad signaling from tumor suppression to tumor promotion.

Notably, alcohol and its metabolic derivatives have been shown to activate the MAPK pathway, and this was reported to be crucial for collagen formation in HSCs[212]. Alcohol-dependent activation of the MAPK pathway depended on the type of hepatic cell and their physiological state, duration of alcohol exposure, and the type of agonist[212]. With specific reference to KCs, TNF-α production and release was linked to chronic alcohol exposure and LPS-induced activation of p38 MAPK[212]. Similarly, LPS-induced activation of ERK and p38 MAPKs was shown to be responsible for liver injury[213]. While elucidating how alcohol and its metabolic derivatives activate the MAPK pathway, Yao *et al*[214] showed that CD14-mediated LPS recognition of TLR4/MD-2 complex mediates TNF-α release secondary to MAPK activation. To stoke more arguments and perhaps stimulate search for explanation, it was reported that activation of p38 MAPK is indispensable for hepatocyte proliferation, while sustained activation of the MAPKs reverses this effect[215,216]. The TGF-β/Smad and the MAPK signaling pathways may be crucial in the initiation and progression of ALD and fibro-hepatocarcinogenesis. It is apparent that a signaling loop possibly involves LPS/TLR4/MD-2/TNF-α-MAPK and TGF-β/Smad crosstalk at multiple levels and plays key roles in ALD as well as recruitment of other signaling pathways implicated in ALD (Figure 3). Some of the signaling pathways recruited into the TGF-β/Smad/MAPK signaling nexus may include but not limited to Spry2[217], EGF and FGF[218], Ras and Wnt/β-catenin[219]. For instance, TGF-β_1 was shown to down-regulate Spry2 in a Smad-dependent fashion[217], meanwhile, Spry2 aids phosphorylation of PTEN and its nuclear accumulation to induce p53-mediated growth arrest[220] which perhaps underlies Spry2-dependent inhibition of HCC cell growth and inhibition of c-Met-induced proliferation and angiogenesis in fibro-hepatocarcinogenesis[218]. But PTEN, which negatively regulates PI3K/AKT signaling, is in turn down-regulated by TGF-β_1[31]. However, it is yet to be determined how alcohol and its metabolic derivatives modulate the tumor suppressor PTEN *via* the TGF-β/Smad/MAPK pathways. Will selective inhibition of TGF-β specific receptors, MAPK-dependent phosphorylation of Smad2/3, TNF-α/TNF-αRI in ALD enhance PTEN expression to halt ALD and the risk of fibro-hepatocarcinogenesis?

CONCLUSION

The structural and functional integrity of the liver is anchored on three pillars: effective modulation of inflammation, oxidative/nitrosative stress, and the innate and adaptive immune systems. From the foregoing, it appears that alcohol and its metabolic derivatives disrupt these three cardinal hepatic functions through reprogramming the functions of hepatic cells to favor ALD and fibro-hepatocarcinogenesis progression through concerted interplay of LPS/TLR4/MD-2/TNF-α-MAPK and TGF-β/Smad signaling. Consequently, alcohol primes the liver to diverse irritants and also increases the sensitivity of hepatic cells to inflammatory nosae derived from non-alcohol sources such as those from co-morbidity factors. This may underlie the spectrum of the pathological features of ALD and other liver disorders, in view of the fact that alcoholics and non-alcoholics alike have in one way or the other been exposed to alcohol and its metabolic derivatives once in their life time either through *de novo* biosynthesis of alcohol from food or from some endogenous substrates under conditions of low oxygen tension such as hypoxia.

Etiologically, metabolic derivatives of alcohol as well as alcohol-dependent alteration of gut microbiome, derangements of gut wall, increased production of PAMPs such as LPS and their subsequent leakage into portal circulation, certainly are the significant alcohol-induced disrupters of inflammatory, innate and adaptive immune regulation within the gut-liver axis. Alcohol-induced liver inflammation, liver fibrosis, and cirrhosis leading to increased risk of HCC may not follow a specific temporal sequence but could follow a multi-dimensional pattern determined in part by the existence of non-alcohol co-morbidity factors.

Mechanistically, alcohol and its metabolic derivatives provide a pathological platform for concerted interaction between pro-inflammatory factors (NF-κB, TNF-α, and IL-1β), pro-fibrogenic factors (TGF-β, Smad, MAPK and PAI-1) and recruitment of other signaling pathways such as the PI3K, Sprouty2 in a TGF-β/Smad-dependent fashion to promote ALD and fibro-hepatocarcinogenesis. TGF-β/Smad/MAPK and their associated cross-signaling nexus must be seen as indispensable in ALD and fibro-hepatocarcinogenesis.

At the research front, it is important that future studies on ALD and fibro-hepatocarcinogenesis focus on experimental models that will permit study of each of the distinct alcohol-derived inflammatory nosae, *i.e.*, AA, MDA, ROS, LPS, AA/MDA-derived protein/DNA adducts and also delineation of nosae-specific pathways, while at the same time excluding overlap

from co-morbidity factors. This will certainly further expand our understanding and discourse on ALD and fibro-hepatocarcinogenesis to guarantee informed prognosis, diagnosis and treatment.

Therapeutically, TGF-β receptor inhibitors, Smad3L-specific inhibitors, MAPK-specific inhibitors, TNF-α/TNF-αRI-specific inhibitors as well as gut-specific therapeutic strategies must feature prominently in therapies designed for ALD and fibro-hepatocarcinogenesis.

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TOPIC HIGHLIGHT

2016 Cirrhosis: Global view

Molecular changes in hepatic metabolism and transport in cirrhosis and their functional importance

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Abstract

Liver cirrhosis is the common endpoint of many hepatic diseases and represents a relevant risk for liver failure and hepatocellular carcinoma. The progress of liver fibrosis and cirrhosis is accompanied by deteriorating liver function. This review summarizes the regulatory and functional changes in phase Ⅰ and phase Ⅱ metabolic enzymes as well as transport proteins and provides an overview regarding lipid and glucose metabolism in cirrhotic patients. Interestingly, phase Ⅰ enzymes are generally downregulated transcriptionally, while phase Ⅱ enzymes are mostly preserved transcriptionally but are reduced in their function. Transport proteins are regulated in a specific way that resembles the molecular changes observed in obstructive cholestasis. Lipid and glucose metabolism are characterized by insulin resistance and catabolism, leading to the disturbance of energy expenditure and wasting. Possible non-invasive tests, especially breath tests, for components of liver metabolism are discussed. The heterogeneity and complexity of changes in hepatic metabolism complicate the assessment of liver function in individual patients. Additionally, studies in humans are rare, and species differences preclude the transferability of data from rodents to humans. In clinical practice, some established global scores or criteria form the basis for the functional evaluation of patients with liver cirrhosis, but difficult treatment decisions such as selection for transplantation or resection require further research regarding the application of existing non-invasive tests and the development of more specific tests.

Key words: Liver cirrhosis; Drug metabolism; Transport; Breath tests; Lipid metabolism; Glucose metabolism

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Core tip: Liver cirrhosis is a common endpoint for many hepatic diseases and is accompanied by the extensive gene regulation of cytokines and enzymes

for hepatic metabolism. The resulting organ deficiency complicates treatment decisions, especially regarding transplantation and the resection of hepatocellular carcinoma. This review summarizes the regulatory events involving the metabolism in the cirrhotic liver and puts these events into the context of the non-invasive testing of liver function. This combination can help to better estimate the liver function of individual patients.

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INTRODUCTION

Liver cirrhosis is the common final pathway of inflammatory liver diseases of different origins. In general, it takes many years or even decades to develop the full picture of liver cirrhosis that is associated with the complete destruction of liver architecture (represented by the liver lobes) through bridging fibrosis. Liver cirrhosis *per se* is the main risk factor for hepatocellular carcinoma and leads, if the underlying disease is not treated adequately, to chronic liver failure. Other pathways to liver failure include acute and acute-on-chronic liver failure (Figure 1). Liver failure of various origins necessitates the transplantation of 5500 livers per year in Europe[1].

Chronic hepatitis B and C and alcoholic and non-alcoholic steatohepatitis are quantitatively the most important causes of cirrhosis, the first mainly in sub-Saharan Africa and most parts of Asia and the latter three in more developed countries in Europe and North America[2]. Other less frequent causes of liver cirrhosis comprise autoimmune diseases such as autoimmune hepatitis, primary biliary cirrhosis or primary sclerosing cholangitis and hereditary entities such as Wilson disease, hemochromatosis and alpha-1-antitrypsin-deficiency. Vascular diseases such as Budd-Chiari syndrome or Osler disease rarely cause liver cirrhosis, while right-heart failure is likely a more frequent cause than is commonly assumed[3]. Finally, drug-induced liver injury[4], recurrent biliary obstruction and rare metabolic disorders such as porphyria can lead to liver cirrhosis and decompensation[5].

The development of cirrhosis is a continuous process from inflammation to fibrosis and ultimately cirrhosis and is complicated by decompensation, liver failure and/or hepatocellular carcinoma (Figure 1). It is accompanied by molecular changes in the hepatocytes and other liver cells modulating the inflammatory and fibrosing process itself that also influence the metabolism of endo- and xenobiotics as well as the synthesis of liver-derived proteins[6]. These changes are the result of the up- or down-regulation of the respective genes in the liver cell or changes in translational mechanisms in the cell. Preliminary data from microarray analysis imply that there are distinct molecular differences between the different etiologies of cirrhosis[7,8]. Research over the past 20 years has been focused on examining molecular mechanisms in the liver for the development of methods to block or retard the development of cirrhosis but has also focused on molecular changes in hepatic metabolism to identify additional risks elicited by insufficient liver function. The limited function of the liver in cirrhosis has significant importance and limits therapeutic options in not only chronic liver disease but also in 70% of all hepatocellular carcinomas (intermediate and advanced stages). The molecular changes in cirrhosis can alter the transport and metabolism of drugs and carcinogens as well as endogenous metabolic intermediates and therefore lead to a higher risk of side effects, drug interactions and genotoxic effects as well as to changes in glucose or lipid metabolism. Many previous studies have used animal models (see below), with unclear transferability to humans. Human studies are scarce and mostly include small sample sizes. This review summarizes the existing knowledge and puts the results into the clinically relevant framework of non-invasive metabolic tests and their application in the clinical routine.

ANIMAL MODELS OF LIVER CIRRHOSIS AND THEIR HUMAN COUNTERPARTS

Experimental liver cirrhosis has been induced by bile duct ligation, toxic compounds such as carbon tetrachloride and the generation of fatty liver for almost a century[9-11]. Over the years, various animal models have been developed in rodents that closely reflect relevant human disease entities and their unique differences[12,13]. The pattern of hepatic fibrosis varies with the model used.

Carbon tetrachloride (CCl_4) is one of the oldest and most widely used toxins for the experimental induction of liver fibrosis in laboratory animals but does not currently resemble a clinically relevant human disease[10,14]. Alternatively, thioacetamide can be used as a supplement to the drinking water to induce severe toxic bridging fibrosis[12,13]. Diethylnitrosamine induces toxic fibrosis that mechanistically resembles CCl_4-induced fibrosis. As rodents develop malignancies under treatment with this carcinogen, it is best for those studying fibrosis in the context of hepatocellular carcinoma (HCC)[13].

Several models have been developed to represent biliary fibrosis and cirrhosis. These include surgical common bile duct ligation, which leads to cirrhosis with signs of portal hypertension and ascites[13]. Genetic alterations of the biliary transporters involved in bile formation are reflected by mice with targeted

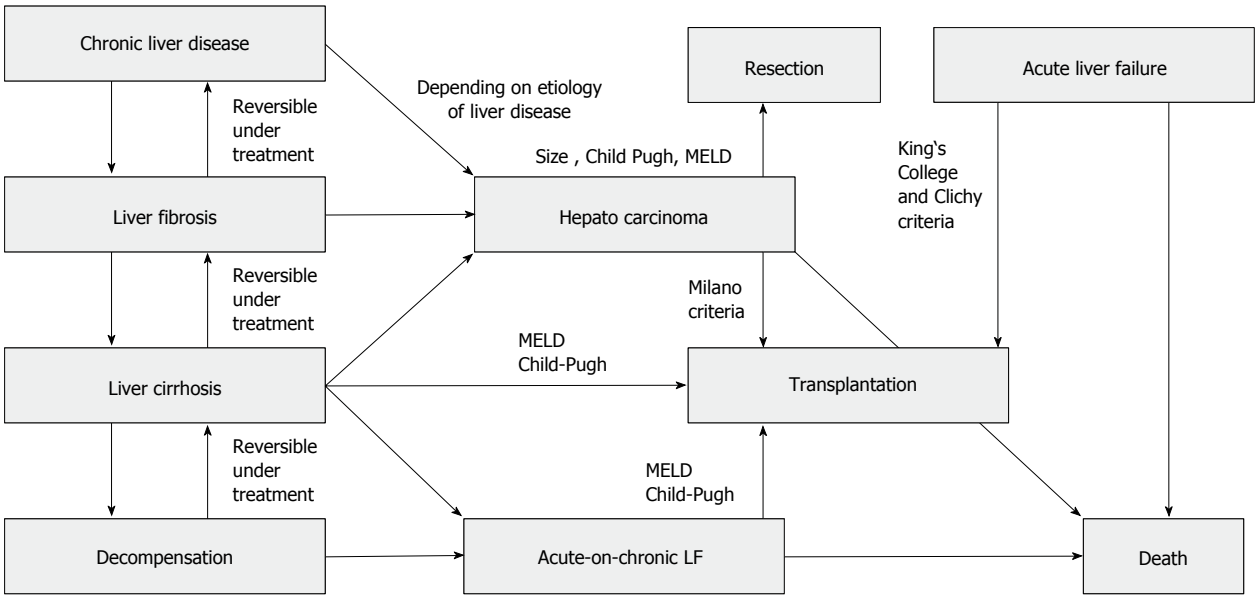

Figure 1 Clinical stages of liver disease. Commonly used scores or criteria are given at the respective treatment boxes (resection or transplantation). LF: Liver failure; for scores and criteria, see Table 2. MELD: Model of end stage liver disease.

disruption of the Mdr2 (*Abcb4*) gene, encoding a canalicular phospholipid flippase (Mdr2$^{-/-}$ mice) that spontaneously develops sclerosing cholangitis with macroscopic and microscopic features of human primary sclerosing cholangitis (PSC) and serves as a model for biliary liver cirrhosis[15,16]. Immunological models include a range from simple heterologous serum application to sophisticated knockout models of autoimmune liver disease[17]. Beyond autoimmune hepatitis models, different spontaneous autoimmune biliary disease mouse models, including the interleukin (IL)-2R$\alpha^{-/-}$ mouse model, have been reported[18].

Non-alcoholic fatty liver disease (NAFLD)-associated cirrhosis currently represents the most frequent liver disease in developed countries. Various rodent models for fatty liver-derived fibrosis are available either nutritionally or based on a genetic modification[19-22]. Among the most widely used models for different stages of the metabolic syndrome including NAFLD are *ob/ob* and *db/bd* mice with genetic alterations in the leptin/leptin receptor pathway and feeding models with high fat (or high fat high fructose). In contrast, the methionine-choline-deficient diet model does not resemble typical human NAFLD in the context of the metabolic syndrome but rather full-blown non-alcoholic steatohepatitis (NASH) with peripheral cachexia[20]. Animal models of alcohol-induced liver disease include a range of different exposure modalities from acute binge ethanol feeding to chronic ethanol feeding (Lieber-DeCarli model)[23].

Genetically humanized mouse models for hepatitis C virus infection are just emerging and have their focus on the immunological pathogenesis rather than on the induction of advanced fibrosis[24]. The same is true for hepatitis B models, which provide clues for understanding host-virus immunologic interactions rather than serve as a disease-specific fibrosis model[25].

As human studies providing data on molecular changes in hepatic metabolism and transport in cirrhosis are very scarce, data from animal models of cirrhosis have been incorporated into this work as far as these models adequately reflect human disease counterparts.

REGULATION

CYP450 isoforms

The human Cytochrome P450s are more than 50 oxygenases present mainly in the liver and the intestine, divided into several families with different substrate specificities. CYP P450 families 1-3 are most important for the metabolism of xenobiotics, including drugs and carcinogens, while the other families catalyze the metabolism of endogenous substrates. A comprehensive review of the structure and function of the CYPs can be found elsewhere[26]. It is very important to note that the CYP450s not only catalyze the detoxification of drugs or carcinogens, but can also promote their carcinogenicity by activating such compounds *via* hydroxylation[27]. Changes in the gene regulation or functional activity of the CYP450s therefore do not have a uniform effect on xenobiotics.

Additionally to this central role of hepatic CYPs in the handling of xenobiotics, the rate-limiting step of bile acid synthesis is mediated by the liver-specific CYP 7A1. Thus, changes in CYP gene regulation also have an impact on bile acid synthesis and lipid metabolism.

CYP isoforms are intensely regulated by nuclear receptors aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator-activated receptors (PPAR) in coordination with phase Ⅱ enzymes and

transporters[28]. Not unexpectedly, inter-individual variations in gene expression are much higher in human samples than in animal livers[29]. Additionally, end-stage livers often exhibit even higher variation than normal livers[30]. Depending on the study design, conflicting data exist regarding the regulation of CYP isoforms in cirrhosis.

In an animal study from 30 years ago, rats with biliary cirrhosis (35 d of bile-duct ligation) showed a reduction in the total protein mass of cytochrome P450 by 45%[31]. In the same study however, toxic post-necrotic cirrhosis in rats (induced by CCl_4) did not lead to a change in the CYP450 protein mass in the diseased livers. This result is in line with recent data regarding non-ascitic animals with cirrhosis, while ascitic cirrhotic rats (assuming a later cirrhosis stage for those animals) had a significant decrease in the total CYP450 protein mass in this newer study[32]. Certainly the differential regulation of specific isoforms of CYP450 contributes to unchanged total protein mass in cirrhosis. CYP450 11B2, for example, was upregulated in rats with fibrotic liver due to CCl_4[33].

In (uninduced) rats, CYP 1A1 is virtually absent, while CYP 1A2 is abundant[32]. Basically, the CYP 1A2 protein mass was decreased to approximately half in cirrhotic rats without ascites; this reduction was intensified to less than 20% in rats with ascites, indicating a dependency on the cirrhosis stage[32]. The mRNA levels correlated well with the protein amount in this study. Interestingly, treatment with benzo[a]pyrene led to the normalization of CYP 1A2 mRNA and protein mass in cirrhotic rats without ascites, while in rats with ascites, a significant decrease remained (albeit lower than in uninduced animals). The same results were obtained for CYP 1A1 in these animals, and all these changes in regulation correlated well with AhR mRNA as well as protein expression[32]. Practically, this means that the ability to have microsomal oxidation (accompanied by possible molecular activation) of carcinogens is preserved in compensated liver cirrhosis (in rats).

Human studies began 20 years ago, when an analysis of 50 end-stage livers showed the differential regulation of CYP isoforms, with the clear basal downregulation of CYP 1A2 as one of the most important activators of xenobiotic carcinogens. In contrast, the CYP 3A protein mass (as the most important isoform for drug metabolism) was only slightly decreased, reaching significance only in non-cholestatic cirrhosis due to hepatocellular diseases, and CYP 2E1 was significantly reduced only in cholestatic cirrhosis[34]. This result implies an interesting isoform-specific regulation in different subforms of cirrhosis. The mechanisms of regulation were diverse and were not restricted to classic translational regulation[35]. These results question an interpretation of gene regulation that rests solely upon mRNA analysis. Very recently, a clear correlation of midazolam clearance (as a surrogate for CYP 3A activity) with the Child Pugh and model of end stage liver disease (MELD) score was shown in 24 patients with end-stage liver disease without any differentiation between cholestatic and non-cholestatic types[36]. Likewise, CYP 1A2, 2C19, 3A4 and 2E1 mRNA were negatively correlated with increasing liver stiffness in additional studies analyzing patients with alcoholic fibrosis[37] or viral hepatitis[38,39]. Combined, these data support the hypothesis that the expression and function of many CYP isoenzymes declines with cirrhosis progression.

In Chinese patients with cirrhosis based on hepatitis B, CYP 1A2 activity measured *via* phenacetin metabolism was decreased by 91%[40]. This result was accompanied by unchanged sulfotransferase (SULT) 1A1 activity in 159 cirrhotic patients, while a subgroup of 46 patients exhibited normal CYP 1A2 activity and elevated SULT 1A1 activity. Interestingly, in a 2 year follow up, three patients from this latter group, but none from the first group, developed HCC, indicating a higher carcinogenic risk with preserved CYP 1A2 activity[40]. These results may offer a chance for identifying a subgroup of cirrhotic patients prone to HCC but were obtained in a simple metabolism analysis of phenacetin without any determination of the molecular expression levels. Even if these results fit with the theoretical considerations regarding the importance of CYP 1A2 in the activation of carcinogens, they should be confirmed in further studies.

In another Chinese study with human end-stage liver disease samples, almost all CYP isoforms (and especially 1A2, 2E1, 2C19, 3A4 and 4A11) were down-regulated to various degrees[30]. This result was accompanied by the down-regulation of the nuclear receptor PPAR, while CAR, PXR and AhR expression was preserved. The data regarding metabolic enzymes are in line with another study already cited above showing the significant down-regulation of CYP 1A2, 2C19 and 2E1 in advanced fibrosis and cirrhosis in patients with viral hepatitis[39]. However, in this study, CAR, PXR and AhR expression were reduced as well, significantly in contrast to the study above. In both studies, the analysis was restricted to mRNA data, while protein expression and functional data were lacking[30,39].

The down-regulation of CYP 2E1 mRNA in cirrhosis, including alcoholic cirrhosis[30], is interesting as this isoform is up-regulated in acute and chronic alcohol consumption and is implicated in the genesis of alcoholic liver damage due to its ability to produce ROS[41,42]. Obviously, cirrhosis as the end stage of alcoholic disease evens this up-regulation into a more dominant general regulatory event associated with severe cell damage.

CYP 7A1 as the rate-limiting enzyme of bile acid synthesis was shown to be up-regulated in primary biliary cholangitis (PBC) patients, but when determined in end-stage patients with PBC (defined as Child Pugh class C), the investigators observed the significant mRNA down-regulation of this enzyme[43]. This

mechanism of late down-regulation may be associated with protective regulation events in liver cirrhosis (avoidance of intracellular bile acid accumulation in hepatocytes) and is confirmed by an additional study[44]. Interestingly, in this latter study, CYP 8B1 as well as CYP 27 mRNAs were preserved, and CYP 3A4 mRNA was only mildly reduced. PBC patients with a certain CYP 7A1 polymorphism leading to higher protein expression of this isoform in hepatocytes were at risk for a rapid PBC progression in a study of more than 300 Japanese patients[45].

In vitro studies in human hepatocytes showed that PXR not only induces CYP 3A4 in normal cells but also mediates the IL-6-induced repression of this CYP isoform[46]. These results may confer an explanation of CYP 3A4 down-regulation in an IL-6 productive state, such as chronic inflammation and liver cirrhosis, but it needs confirmation in *in vivo* studies. A recent human study implies that microRNA-155, a known regulator of liver inflammation, may contribute to lower CYP 3A4 activity in liver cirrhosis[47], but the data presented in this study are merely descriptive and lack a clear mechanistic explanation.

The effect of liver fibrosis or cirrhosis on CYP450 expression and function outside the liver remains controversial. In a human study with 23 patients with various degrees of cirrhosis, duodenal CYP 3A expression and total midazolam hydroxylation were both reduced to less than 50% of normal control patients[48]. In a pharmacokinetic animal study of ofloxacin, the authors found increased CYP enzyme activity in cirrhotic rats (CCl_4, ethanol and high fat) as a reason for prolonged and reduced bioavailability of the test substance[49]. It must be clarified in additional studies whether these conflicting data are species-specific or relate to the different study designs.

In summary, most CYP isoforms are reduced in expression and activity in advanced fibrosis and cirrhosis. This especially holds true for the important isoenzymes 1A2, 2E1 and 3A4 (for exogenous compounds) but also for CYP 7A1 (for bile acids). One important problem in many studies is that only mRNA data are available for the respective animal model or the human liver disease. Additionally, even protein expression data do not necessarily reflect the enzyme activity *in vivo* in advanced liver disease (see also below).

Phase-Ⅱ metabolism (UGT's, sulfotransferases)

Phase Ⅱ in metabolism of xenobiotics is conferred by several groups of enzymes, the most important of which are uridine diphosphate (UDP)-glucuronosyltransferases (2 families with more than 20 isoforms[50]) and sulfotransferases (13 isoforms in 4 groups[51]). The ultimate goal of phase Ⅱ metabolism is the solubilization of metabolites in water and thereby the potential for excretion in urine and bile. Nevertheless, sulfatation by sulfotransferases can also potentiate the genotoxic effect of a certain carcinogen. In general however, these metabolites are no longer toxic or carcinogenic, and therefore phase Ⅱ-metabolism is the final step of detoxification (before transport into urine or bile). Not surprisingly, both groups of enzymes are regulated coordinately by the nuclear receptors AhR, CAR, PXR and PPAR[28,52] together with phase Ⅰ and transporters, and this principle also holds for human liver disease[39]. In the latter study, coordination between nuclear receptors and metabolic enzymes was even stronger in severe liver disease (METAVIR score 3-4 in patients with mainly HCV) than in mild liver disease, indicating increasing cross-talk between transcription factors[39]. Human SULT 2A1, an important sulfotransferase for endogenous compounds including bile acids, has been shown to be regulated by the retinoid-related orphan receptors RORα and β[53]. For UDP-glucuronosyltransferase (UGT) 1A7, a clear association of low-activity genotypes with cirrhosis, functional hepatic impairment and HCC was shown[54], indicating the importance of detoxification by this isoenzyme in the pathophysiology of chronic liver disease.

Increasing amounts of deposited cholesterol in the livers of rats fed with high cholesterol diet, with final development of fibrotic steatohepatitis, led to a progressive down-regulation of the mRNA expression of SULT 2A1 and UGT 1A1 as well as UGT activity, most likely due to the parallel down-regulation of PXR and CAR[55]. In a rat model of toxic fibrosis/cirrhosis (treatment with thioacetamide), major UGT isoforms were up-regulated, but the enzyme activity was unchanged[56]. In CCl_4-cirrhotic rat livers, however, UGT protein expression was completely preserved while enzyme activity was not measured[57]. In biliary cirrhotic rats, we also found the protein content of UGT 1A isoforms unchanged, but the enzyme activity of both UGT and SULT isoforms was clearly reduced[58]. As *Mrp2* expression (the main transporter of glucuronidated and sulfated metabolites) is extensively down-regulated in biliary cirrhosis, these results can be interpreted as end-product inhibition of UGT 1A activity *via* lack of efflux[58].

Zollner *et al*[44] investigated the mRNA expression of a few UGT and SULT isoforms (UGT 2B4 and 2B7 and SULT 2A1) in 11 PBC patients and found them unaltered. This result is only partially in line with an earlier human study showing the mRNA expression levels of several UGT isoforms (1A4, 2B4, 2B7) to be significantly down-regulated in inflammation but not in fibrotic livers[59]. Later, the same group confirmed unaltered UGT isoenzyme mRNA in advanced fibrosis or cirrhosis in viral hepatitis[39]. In a recent human study, the authors analyzed UGT and SULT expression along with NAFLD from steatosis *via* NASH to cirrhosis[60]. In this comprehensive study, the mRNA expression levels of numerous UGT and SULT isoforms were almost invariably preserved in cirrhosis. Protein expression of the tested UGT isoforms (1A1, 1A6, 1A9

and 2B10) was also very similar in normal, steatotic and cirrhotic tissue, with a significant down-regulation in cirrhosis only for 1A6. SULT isoenzymes protein expression levels (analyzed for 1A1, 1C4 and 2A1) were significantly up-regulated for SULT 1C4 (important for endocrine metabolism) and down-regulated for 2A1 in cirrhotic NASH. The significance of these specific regulatory events on enzyme activity or hepatic metabolism must remain open as in this study, UGT activity was unchanged in all NASH patients, and SULT activity was significantly reduced in fatty and cirrhotic NASH patients[60]. In another study analyzing the SULT isoenzymes 1A1, 2A1, 1E1 and 1A3 in fatty liver disease (both NASH and ASH), the authors showed a clear reduction of SULT 1A1, 1A3 and 1E1 activity and protein expression correlating to the extent of fatty liver disease[61]. This down-regulation was pronounced in alcoholic cirrhotic patients, where additionally SULT 2A1 activity was reduced. In general, this study found a clear reduction in the protein expression and function of SULT isoenzymes with the progression of liver disease[61]. In contrast, SULT 1A1 activity was unchanged in cirrhosis in a study analyzing phenacetin metabolism, while cirrhotic patients with elevated SULT 1A1 activity (along with preserved CYP 1A2 activity) were at higher risk for HCC (see above[40]). These partially conflicting data may indicate on the one hand high interindividual differences and on the other hand the decoupling of molecular expression and activity of isoenzymes during advancing fibrosis. Additionally, a decrease in SULT expression or activity can be modified by sulfatase activity, which is known to be reduced in cirrhosis as well[62].

In general, UGT and SULT expression seem to be largely unchanged in advanced fibrosis and cirrhosis. However, with regard to the enzyme activity of these two important phase Ⅱ enzymes, we are facing a serious methodological problem. Most studies (especially in humans) have used in vitro methods with defined substrates to determine the activity of the respective isoenzymes[60,61]. In these isolated test situations, the pure enzymatic activity of each specific isoenzyme can be determined accurately and is preserved for most isoenzymes. In contrast, data from rat *in vivo* experiments indicate that in the cirrhotic liver, where the transport of some phase Ⅱ metabolites is impaired (see below), the phenomenon of end product inhibition can occur[58]. As phase Ⅱ metabolism is crucially linked to transporter-mediated export into bile, these results in a reduced *in vivo* activity of phase Ⅱ isoenzymes despite preserved *in vitro* activity. The validity of *in vitro* data is therefore in question, which in turn indicates the increasing importance of *in vivo* test systems for hepatic metabolism in health and disease.

Transport

Functional changes in enterohepatic transport systems have been described in experimental liver disease and specific human disease entities[63,64].

Basolateral import transporters of the liver are down-regulated in inflammatory and cholestatic conditions[63]. In human cholestatic liver disease, decreased Na-taurocholate co-transporting polypeptide (NTCP) (SLC 10A1), mRNA and protein levels have been observed in PBC patients with stage Ⅲ and Ⅳ disease[65,66] as well as biliary atresia[67]. Reduced organic anion-transporting polypeptides (OATP1B1) and OATP1B3 mRNA and protein expression have also been described in the later stages of PBC[65,66]. In line with these findings, OATP1B1 down-regulation can be observed in other cholestatic conditions such as PSC[68]. Remarkably, the expression of NTCP is only reduced in PBC stage Ⅳ (cirrhosis), whereas OATP1B1 is diminished at an earlier stage Ⅲ[65,68]. The down-regulation of NTCP (SLC 10A1) and OATP1B1 may not only contribute to impaired hepatic bile salt uptake in the advanced stages of cholestatic liver disease but could also represent a defense mechanism that is partially limiting the accumulation of potentially toxic bile salts[65]. As another line of defense, the compensatory upregulation of basolateral escape transporters such as the multidrug resistance-associated proteins MRP3 (ABCC3) and MRP4 (ABCC4) is already induced at a precirrhotic PBC stage, while canalicular ATP-dependent export pumps remain stably expressed in the cirrhotic stage[44,65,66,69].

Canalicular transport systems of the liver are less tightly regulated in inflammatory and cholestatic conditions. For Multidrug resistance-associated protein 2 (MRP2, ABCC2), decreased immunostaining has been described in a subset of PBC patients with stage Ⅳ disease and progressive cholestasis[70]. Similarly, decreased MRP2 (ABCC2) mRNA levels have also been observed in PSC patients and patients with poorly drained obstructive cholestasis[68,71]. In the latter study, the mRNA levels of MRP2 (ABCC2) and bile salt export pump (BSEP, ABCB11) were decreased in poorly drained compared to well-drained patients, who were at the levels of control subjects. Immunostaining of MRP2 (ABCC2) and BSEP (ABCB11) at the canalicular membrane domain were fuzzy to varying degrees in the specimens obtained from poorly drained cholestatic liver but linear and intense in the liver of well-drained patients and control subjects, correlating with impaired bilirubin conjugate and bile acid secretion[71].

The down-regulation of hepatic transport systems has also been observed in patients with non-cholestatic chronic inflammation of the liver such as hepatitis C infection. Together with the expression levels of nuclear receptors as the transactivators, the mRNA levels of various transporter genes, including NTCP (SLC10A1), OATP1B1, BSEP (ABCB11) and MRP2 (ABCC2), are decreased depending on the stage of fibrosis, with an approximately 50 % decrease between F3 and F1 patients[72]. In another study investigating viral hepatitis C patients, inflammatory cytokines such as tumor necrosis factor (TNF)α have been found to be

increased with fibrosis stage F3, while transporters including OATP1B1 were decreased[38]. Additional cell culture experiments have also demonstrated a functional contribution of interleukin (IL)-1 and -6, which was most prominent for NTCP (SLC 10A1).

The functional consequences of decreased transporter expression during the progression of fibrogenesis have been studied in rats with experimental biliary cirrhosis[58]. A significant down-regulation of canalicular multidrug-resistance transporters, including Mrp2 (Abcc2) and Bcrp (Abcg2), has been detected, while the biliary excretion of a radiolabelled food-derived carcinogen into the bile was significantly decreased. Of note, the mRNA and protein expression of MRP2 (ABCC2) was only moderately decreased in human livers with alcoholic cirrhosis, whereas BCRP (ABCG2) was increased[73]. Therefore, the potential contribution of decreased carcinogen defense transporters to the increased hepatic and extrahepatic incidence of cancers in cirrhosis patients remains to be evaluated in more detail. Additional findings for hepatic uptake systems have been obtained in rat liver perfusion experiments. Here, a linear relationship was found to exist between the histopathologic fibrosis index and the hepatic extraction ratio of 3H-taurocholate[74].

In summary, changes in transporter expression in cirrhosis fit into the "cholestatic paradigm"[75] of transporter regulation. Cirrhosis also represents a cholestatic state with the intracellular accumulation of bile acids in hepatocytes. Consequently, import transporters (at the basolateral membrane) are downregulated, and export transporters (especially basolaterally) are simultaneously upregulated. At the canalicular membrane, the regulation events are less uniform and also depend upon the stage and pathogenesis of fibrosis and cirrhosis.

In clinical practice, decreased hepatic transport and metabolic function may be critical for decision making in critically ill patients or those undergoing hepatic intervention or surgery. Methods to assess hepatic function quantify the abundance and functional integrity of the basolateral uptake and canalicular export systems described above. The indocyanine green (ICG) disappearance rate reflects a direct non-invasive measure of the actual functional state of these hepatic transport systems at the time of assessment[76-78]. Albumin-bound water-soluble ICG, which is not metabolized by hepatocytes[79], is selectively taken up by the basolateral uptake systems NTCP (SLC10A1) and OATP1B3 and is later excreted unchanged into the bile by the canalicular MRP2 (ABCC2) transporter[80]. At the basolateral membrane, OATP1B1 and OATP2B1 are both inhibited by ICG[80]. ICG clearance thus reflects the overall hepatic uptake, and excretory function and can be used to assess liver function in patients with chronic liver failure and as a prognostic factor in critically ill patients[76]. However, a delayed residual ICG excretion indicates an additional transcellular pathway, which can be blocked by colchicines[81]. This might be an explanation for ICG plasma disappearance that occurs in humans during the anhepatic phase of orthotopic liver transplantation, possibly hampering the validity of the test[82].

Although less frequently used in clinical practice today, hepatobiliary radiotracers such as 99mTc-mebrofenin and 99mTc-N-pyridoxyl-5-methyltryptophan (99mTc-PMT) share a transporter spectrum that is partially overlapping with ICG, which involves OATP1B1 and OATP1B3 for basolateral uptake[80,83,84]. 99mTc-mebrofenin and 99mTc-PMT excretion into bile canaliculi is facilitated by the canalicular ATP-dependent export pumps MDR1 (ABCB1) and MRP2 (ABCC2)[83,84], which contribute to the visualization of biliary structures in clinical scintigraphy.

Changes in transporter expression in chronic liver disease associated with fibrosis or even cirrhosis also have implications for MRI-based imaging. MRI contrast agents are taken up into and excreted out of hepatocytes by the same transporters of the OATP and MRP family. Experimental cirrhosis in rats is associated with the decreased entry of Gd-BOPTA into hepatocytes in a radioactivity distribution compartment model[85] in agreement with the reduced expression of Oatp transporters in experimental cirrhosis[86]. Although the entry of contrast agent into hepatocytes was lower in cirrhotic than in normal livers, the accumulation of Gd-BOPTA was higher in cirrhotic livers because biliary excretion was totally abolished[85], again correlating with decreased Mrp2 (Abcc2) expression in previous studies. Additionally, the Gd-EOB-DTPA uptake in hepatocytes is strongly affected by liver function[87]. Gd-EOB-DTPA-enhanced MRI and the assessment of relative enhancement during the hepatobiliary phase may serve as a useful image-based test in liver imaging for determining regional and global liver function[88].

Lipid and glucose metabolism

Interaction and changes in hepatic lipid and glucose metabolism are important for the etiology and progression of non-alcoholic steatohepatitis[89]. These changes are not confined to the liver, and they contribute to the development of liver cirrhosis rather than being the result of cirrhosis development. Insulin resistance, increased synthesis and the release of free fatty acids and changes in the production of leptin, adiponectin and interleukins 1 and 6 are the central players in NAFLD and NASH-dependent liver fibrosis[90,91], and these changes are connected to excessive fat accumulation in obese NASH patients[92].

However, studies investigating changes in lipid and glucose metabolism in liver cirrhosis are rare. Insulin resistance is also an important hallmark in liver cirrhosis, but here it is a catabolic disease associated with muscle wasting, anorexia and weight loss. Twenty years ago, a receptor/postreceptor dysfunction was already postulated as the explanation for the glucose

metabolism disturbances and malnutrition found in cirrhotic patients, based on altered membrane lipid composition, hyperinsulinemia and a lack of liver-derived humoral factors[93]. Protein synthesis in general is disturbed in liver cirrhosis[94], intensifying ER stress and organ failure[95], but the changes in glucose and lipid metabolism are more complex and are individually very diverse depending on the general metabolic status. This concept is also illustrated by the results of a microarray analysis of fibrosis progression in hepatitis c patients. In this study, the amino acid metabolism enzymes were more severely and uniformly down-regulated than were the glucose and lipid metabolism enzymes[6].

Patients with cirrhosis were shown to have a lower energy intake and a higher resting energy expenditure, higher fasting leptin and higher insulin resistance than controls[96]. In a follow-up study with 42 cirrhotic patients, the same group found increased postprandial glucose, insulin and glucagon-like peptide 1 responses and reduced postprandial ghrelin. Interestingly, in this latter study, these metabolic changes were related to delayed gastric emptying and prolonged small bowel transit[97], and a high proportion of these patients suffered from gastrointestinal symptoms. Additionally, cirrhotic patients showed increased rates of gluconeogenesis but lower net hepatic glycogenolysis[98]. These effects explain the higher insulin resistance and the diminished reaction to hypoglycemia in cirrhotic patients. Hyperinsulinemia in cirrhotic patients has also been linked to an increased pancreatic beta-cell sensitivity to glucose[99], again an extrahepatic metabolic effect of liver cirrhosis. In the explanation of higher circulating plasma levels of enzymes (*e.g.*, insulin or glucagon) or proteins in general, the decreasing hepatic extraction capacity as represented by, for example, the asialoglycoprotein-receptor certainly plays a role[100] but must be quantified individually[99]. However, again, effects such as this may form a basis for another non-invasive test of a part of liver function, in this case the extraction of circulating proteins from the portal blood[101-104].

Fibroblast growth factor (FGF) 15/19 acts as a FXR-activated negative feedback regulator that signals from the intestine to the liver to repress bile acid synthesis and has recently been recognized to regulate energy homeostasis and lipid metabolism (Jahn and Geier, Mechanisms of enterohepatic Fibroblast Growth Factor (FGF) 15/19 signaling in health and disease, manuscript submitted). Intestinal FGF19 has emerged as a novel endocrine regulator of hepatic bile salt and lipid metabolism, and an impaired hepatic response to FGF19 may contribute to the dysregulation of lipid homeostasis in NAFLD patients[105]. Although no data on FGF19 expression and signaling exist in human liver cirrhosis, recent data from mouse experiments indicate that activated ileal FGF15 may contribute to HCC development in the context of chronic liver injury and fibrosis[106].

Many associations between cytokine regulation and metabolism have been identified. The level of the adipokine resistin was increased in 57 cirrhotic patients and correlated negatively with hepatic glucose production and positively with circulating free fatty acids and TNF-α levels, implicating an effect in glucose and lipid metabolism. However, resistin was not associated with the degree of insulin resistance after transplantation; the resistin levels remained unchanged, while the insulin resistance was significantly improved[107]. Adiponectin has also been shown to be elevated in liver cirrhosis without any etectable correlation with the parameters of lipid or glucose metabolism or proinflammatory cytokines[108]. So far, the overall influence of these cytokine regulatory events on lipid and glucose metabolism is unclear, and a causal relationship has not continuously been shown. However, it is obvious that altered cytokine profiles in liver cirrhosis contribute to systemic alterations in lipid and glucose metabolism that concern many extrahepatic sites, such as the pancreas, the gut and the muscle tissue. Still, the exact mechanisms of altered lipid and glucose metabolism during liver cirrhosis deserve further research.

In addition to the general changes in cirrhotic livers, disease-specific metabolic events must be considered in these patients. As such, hepatitis B virus (HBV) infection alters bile acid and cholesterol metabolism as a consequence of impaired NTCP-mediated bile acid uptake into hepatocytes[109]. Using human liver-chimeric uPA/SCID mice (SCID - severe combined immunodeficiency) and human liver biopsies from HBV patients, Oehler *et al*[110] showed that nuclear FXR localization and SHP expression are decreased with chronic HBV infection, leading to relevant expression changes in genes involved in bile acid synthesis as well as cholesterol uptake and synthesis. The metabolic consequences for patients with chronic HBV infection and particularly end-stage liver disease remain to be determined. The regulatory events of liver cirrhosis described in this chapter are summarized in Figure 2.

BREATH TESTS FOR LIVER METABOLISM AND FUNCTION

Several functions of hepatic metabolism can be monitored non-invasively using specific breath test analysis. The determination of pCO_2 in breath following the ingestion of a meal was the first application of a breath analysis in hepato-gastroenterology in the early twentieth century[111-113]. Because of their minimally invasive nature and feasibility, breath tests were attractive for their clinical use but had no clinical applications for almost 40 years. Later on, a large variety of breath tests were introduced as "no-touch" functional diagnostic tests and are currently performed to investigate gastrointestinal motility and liver disorders[114]. Typical stable isotope breath tests

Table 1 Typical clinical applications for the quantitative assessment of hepatic function by means of ^{13}C-labeled compounds as breath test substrates

Investigated function	^{13}C-labelled compound
Bile acid malabsorption	Glycocholic acid
Hepatic microsomal function (*CYP P450*)	Aminopyrine, methacetin, phenacetin caffeine, diazepam, erythromycin
Hepatic cytosolic function (*galactokinase*)	Galactose, phenylalanine
Hepatic mitochondrial function (*α-keto acid dehydrogenase complex*)	Methionine, octanoate, α-ketoisocaproic acid

for liver function are listed in Table 1.

Liver function tests using unlabeled and labeled (^{13}C, ^{14}C, D, ^{15}N) compounds as marker substances play an important role for the management of patients with chronic liver disease. Chronic liver diseases comprise pathomorphological features such as necrosis, inflammation, fibrosis, changes of intra- and extrahepatic blood flow and impaired function. These changes result in the typical clinical manifestations of complications governing overall patient outcome, whereas the degree of functional impairment has been described as a stronger predictor for the clinical outcome than are histological changes in patients with chronic hepatitis C infection[115]. Indices for the prediction of survival are essential tools for assessing prognosis, establishing priority for liver transplantation and identifying those at high risk for developing complications due to disease progression or following interventions[116-118]. Determining the hepatic reserve is essential for assessing the prognosis, predicting decompensation and organ allocation[119,120]. Decision-making in the treatment of patients with chronic liver disease focuses on the early identification of these patients[118,120]. For the non-invasive evaluation of "quantitative liver function" exogenous/xenobiotic or natural liver, specific test substances have been introduced to specify partial function, such as hepatic blood flow (HBF) by ICG clearance or cholate clearance, hepatic plasma flow by sorbitol elimination capacity, cytosolic liver function by (^{13}C) galactose elimination capacity, mitochondrial function by alpha-ketoisocaproic acid or ^{13}C-methionine breath test (MeBT), hepatic cytochrome P450 function by ^{13}C aminopyrine (CYP 2C19, 2D6) (ABT), caffeine test or ^{13}C-methacetin (CYP 1A2) (MBT) breath test or lidocaine/monoethylglycinexylidite (CYP 3A4) (MEGX) test[121-134]. The dual cholate test is a novel oral (D4-cholate) and intravenous (^{13}C-cholate) simultaneous function test that quantifies clearance from the systemic circulation, portal circulation and portal systemic shunting[134].

Hepatic clearance (in mL of plasma/min/kg of substance cleared) can be flow or functional liver cell mass-dependent (comprising extraction and metabolic efficiency of hepatocytes) or both and is given by[134,135]:

clearance = [c (arterial) - c (hepatic venous)]/c (arterial) × hepatic blood flow

with [c (arterial) - c (hepatic venous)]/c (arterial) defined as first pass hepatic extraction E, calculated from the concentrations of the substance measured in the arterial and hepatic venous blood[136]. For substances with high extraction, which is facilitated by high affinity transport systems, *e.g.*, for orally administered bile acids[137], the membrane sodium-dependent bile acid transporter and organic solute transporter (OST-α/OST-β in enterocytes, the Na-dependent taurocholate cotransporter (NTCP, SLC10A1) and the Na-independent superfamily of organic anion transporting polypeptides (OATP) at the basolateral membrane of hepatocytes, elimination half-lives are in the range of minutes, and the clearance is close to HBF. If flow-dependent substances are administered, such as ICG[138] or lidocaine[139], then the hepatic extraction of ICG in normal controls measured by hepatic venous catheter is 0.7-0.9. In patients with liver diseases, it is reduced, with values < 0.3.

Test substances with E < 0.25, such as the CYP-metabolized xenobiotics aminopyrine or diazepam, are only extracted to a small amount during liver passage, *i.e.*, the hepatic disposition of the substance is determined only by the metabolic capacity of the liver and not by HBF. Interestingly, for most substrates frequently used for the assessment of hepatic biotransformation function (methacetin, aminopyrine), the transport mechanisms are not well described. For example, erythromycin applied as a ^{13}C-labeled substrate in a breath test for hepatic CYP 3A4 activity inhibits OATP1B1 and OATP1B3[140], is transported by OATP1B1 and is a substrate for MRP2 (see also ICG transport above[135]). Again, transport function alters erythromycin metabolism, showing a close relationship with hepatocyte metabolism and transport in humans as well[141]. This interrelationship must be implemented in the interpretation of the ^{13}C erythromycin breath test. The biotransformation of drugs is reduced in patients with severe liver diseases, whereas the microsomal monooxygenase system is the most affected. The well-known reduction of total cytochrome P450 protein in patients with liver cirrhosis[142,143] could be characterized in detail by George and co-workers for specific CYP subfamilies in patients with cholestatic (primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia, idiopathic cholestasis) and non-cholestatic liver cirrhosis (auto-immune hepatitis, alcoholic cirrhosis, chronic viral hepatitis) in different Child Pugh stages (see above[34]). Furthermore, the CYP1A2 protein amount and catalytic activity is significantly and homogenously reduced in both cholestatic and non-cholestatic liver cirrhosis compared to a control group, which serves as a pathobiochemical basis of the frequently used ^{13}C-methacetin breath test[34,144-147]. It is important to note that the activity of the NADPH-cytochrome P450 reductase is not

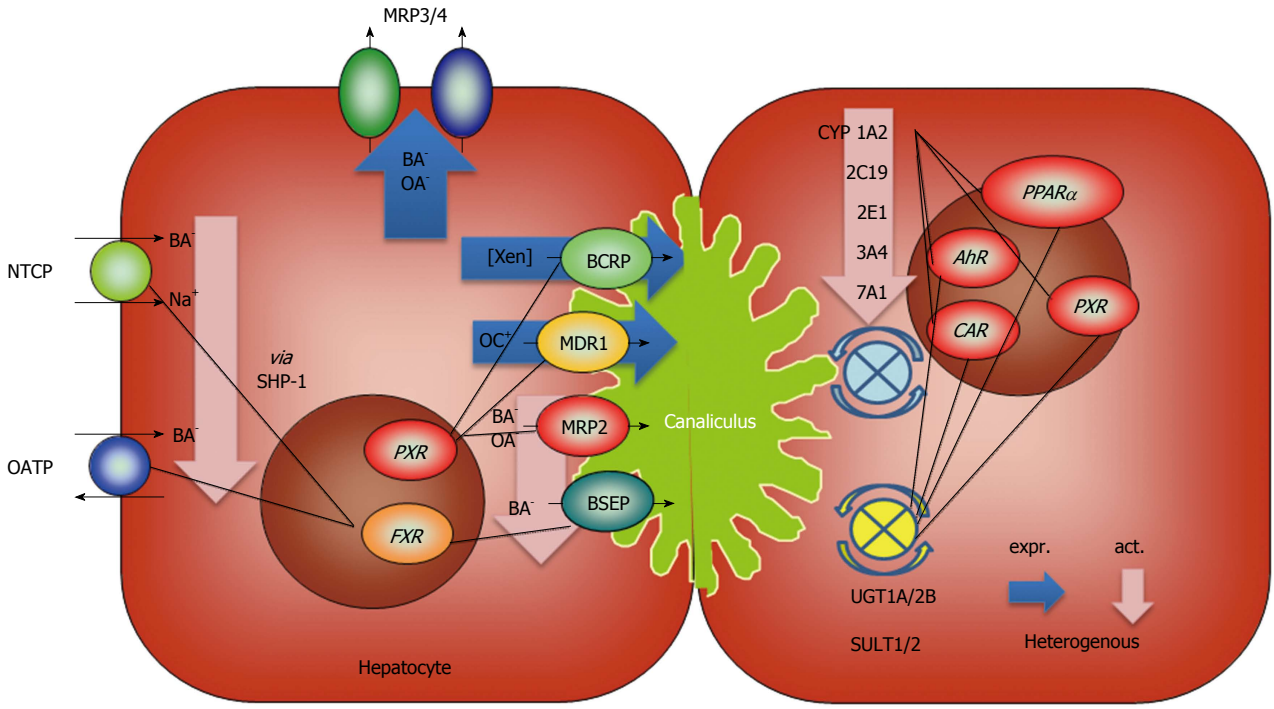

Figure 2 Regulatory events in hepatocytes in liver cirrhosis. In the left hepatocyte, the regulation in transport proteins (phase 0 and phase Ⅲ) is shown, and in the right hepatocyte, regulation in phase Ⅰ and phase Ⅱ metabolism is summarized. Large arrows show the tendency of protein expression and/or activity for the respective single enzyme/transporter or groups of enzymes/transporters (up- or downregulation or no change). Central nuclear receptors for each group of enzymes are additionally shown. Details and source of the data are given in the text. expr.: Expression; act.: Activity; BA: Bile acids; OA: Organic anions; OC: Organic cations; Xen: Xenobiotics; NTCP: Na-taurocholate co-transporting polypeptide; OATP: Organic anion-transporting polypeptide; UGT: Uridine-diphosphate-glucuronosyltransferase; SULT: Sulfotransferase; MRP: Multidrug resistance-associated protein.

decreased in patients with liver cirrhosis, and thus the reduction of specific CYP isoforms in hepatocytes for the selective change of the mixed functional monooxygenase activity is causative[34].

In summary, transport systems are an important component of well-established flow and liver cell mass-dependent liver function tests. The involvement of multiple processes, including substance uptake in the intestine and the hepatocyte, metabolism, and excretion from the hepatocyte indicate that the (patho)-physiological interpretation of a liver function test is multilayered and might influence the clinical value of a single test[135].

CONCLUSION

Liver cirrhosis is a serious disease with far-reaching changes in gene expression and function. The impairment of hepatic function has consequences for the treatment and prognosis of patients. These consequences mainly comprise the resection of hepatocellular carcinoma (or other tumors including metastasis) and an indication for liver transplantation. Additionally, decisions about medical treatment must take impaired liver function into account (dose adjustment), and the altered metabolism of xenobiotics including carcinogens can have an impact on toxic or carcinogenic effects in the body.

Metabolism in the liver represents a coordinated sequence of enzymatic steps: (1) extraction of compounds from the portal blood is followed by uptake into the hepatocyte (phase 0, basolateral import); (2) oxygenation/activation of compounds (phase I, CYP 450); (3) glucuronidation or sulfation, and rarely acetylation, methylation or conjugation to glutathione (phase Ⅱ, UGT, SULT and others); and (4) secretion *via* the basolateral or canalicular membrane to the caval blood or the bile (phase Ⅲ, basolateral or canalicular export transporters).

The close connection between these steps is also embodied by the coordinated regulation of these metabolic steps by central nuclear receptors, which is even stronger in diseased livers[28,39]. The data summarized in this review show the down-regulation of important CYP 450 isoforms[34,35] and basolateral import transporters[65,66]. Phase Ⅱ enzymes (UGT, SULT) are mostly preserved in their expression[39,44,60], but data from animal *in vivo* experiments point to a reduction of enzymatic function[58]. Lipid or glucose metabolism is individually altered as a result of cytokine regulation, differential enzyme expression and basic metabolic status but is in general characterized by hyperinsulinemia, insulin resistance and catabolism[96,98]. These data show the complexity of metabolic processes and their regulation in cirrhosis.

Experimental data clearly show that every metabolic step can influence the preceding steps of metabolism in their functional capacity or differentiation. In a

Table 2 Established scores or criteria for treatment decisions in hepatology, some criteria are used with different parameters in different countries

Name	Parameters	Used for
MELD[163]	INR, creatinine, bilirubin	CLF, LTX, resection
Child Pugh[164]	Prothrombin time, bilirubin, ascites, encephal., albumin	CLF, resection
King's College[165]	Paracetamol-ALF: pH, INR, creatinine, encephal	ALF, LTX
	Non-paracetamol-ALF: INR, age, bilirubin, timing of jaundice, etiology	
Clichy[165]	Age, factor V, encephalopathy	ALF, LTX
Milano[166]	Size and number of tumor nodules	HCC, LTX

INR: International normalized ratio; CLF: Chronic liver failure; ALF: Acute liver failure; encephal.: Encephalopathy; LTX: Liver transplantation; HCC: Hepatocellular carcinoma.

study in Gunn rats (an animal model for Crigler-Najjar syndrome), the lack of UGT1 isoforms significantly changed the metabolic ratio of phase Ⅰ metabolism conferred by CYP isoforms[148]. The influence of a single transporter function on hepatic metabolism is best exemplified by MRP2. MRP2 represents a transporter for organic anions[149] but also transports amphipathic compounds in co-transport with glutathione[150,151]. The genetic loss of MRP2 expression forms the basis for the rare Dubin Johnson syndrome[152], but acquired MRP2 deficiency is common in cholestatic diseases, including liver cirrhosis[72,73]. In Mrp2 knockout mice but also in patients with a MRP2 polymorphism, metabolism of erythromycin was altered independently from CYP 3A4 expression and function[141]. In animal studies, the pronounced down-regulation of the canalicular organic anion transporter MRP2 is supposed to be responsible for the pronounced functional inhibition of phase Ⅱ enzymes[58] despite preserved enzyme expression. The up-regulation of Mrp3 and 4 at the basolateral membrane can obviously only partially compensate for this acquired Mrp2 deficiency. Phase Ⅱ metabolism is extremely important in the detoxification of endogenous and exogenous toxicants, including carcinogens. At least in rats, the accumulation of metabolized but also activated carcinogens in the liver and in other organs as a consequence of genetic or acquired Mrp2 deficiency was shown and may have consequences for the toxic and carcinogenic effects of xenobiotics[58,153]. However, species differences can complicate the interpretation of these results. Hepatic BCRP, a transporter with overlapping substrate specificity, can compensate for MRP2 deficiency in humans, where it is preserved or even upregulated[73], while in rats, it is down-regulated during liver cirrhosis[58]. As a further complication, extrahepatic expression of these transporters also contributes to metabolism, tissue accumulation and the excretion of toxic compounds. In colonic adenomas, BCRP is more down-regulated in humans than in mice, where the accumulation of a food-derived carcinogen has been shown to promote carcinogen accumulation[154]. The overall effect of impaired liver function the metabolism of toxins and carcinogens is not sufficiently defined and needs further studies, especially epidemiological data[155].

Dose adjustments in patients with liver cirrhosis are also difficult and mostly based on rough calculation of hepatic function with Child Pugh criteria[156]. Many of the tests mentioned above, using either blood or breath samples, exploit pharmaceutical compounds such as midazolam or erythromycin, but no test can be recommended for dosing decisions in cirrhotic patients[114,156].

The summarized data show that in humans, enzymatic functions are difficult to test *in vivo*, and therefore *ex vivo* methods (*e.g.*, microsomal assays) have been used. In these assays, however, the enzymatic function is tested in an isolated manner, leaving out the necessary connection between phases 0 and Ⅲ. For almost all steps in hepatic metabolism, there is a test that can be applied, but the full picture is hidden behind many complex regulatory events. No single test is able to reliably estimate liver function simply because liver function is extremely complex and encompasses many diverse functions. Breath tests have advantages in daily patient care as they are non-invasive, readily available and can be applied *in vivo* in the intact metabolic sequence[114,115,134]. Nevertheless, even these tests are dependent on test substances, and therefore the used test substance and its rate-limiting step determines the value of the test[114]. From a logical point of view, it certainly is useful to combine breath tests with different test substances and different rate-limiting metabolic steps to examine different aspects of liver function in a test panel.

In every-day practice, established global easy-to-measure scores such as MELD or Child Pugh will be used for a first estimation of liver function (Table 2 and Figure 1). The continuing discussion about the efficacy and validity of these scores already shows their limitations[157-160]. We know too little about functional tests in liver cirrhosis, and all available tests only represent parts of the individual's liver function. As a consequence, even applying multiple tests on different aspects of liver function cannot avoid the misjudgment of individual patients[161,162]. If difficult decisions in treatment must be made (*e.g.*, partial liver resection or liver transplantation), the application of two or more complementary breath tests as outlined above may

help in an appropriate classification of liver function to live up to the expectations of clinicians and patients in legitimate treatment decisions.

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TOPIC HIGHLIGHT

2016 Cirrhosis: Global view

Four-dimensional flow magnetic resonance imaging in cirrhosis

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Abstract

Since its introduction in the 1970's, magnetic resonance imaging (MRI) has become a standard imaging modality. With its broad and standardized application, it is firmly established in the clinical routine and an essential element in cardiovascular and abdominal imaging. In addition to sonography and computer tomography, MRI is a valuable tool for diagnosing cardiovascular and abdominal diseases, for determining disease severity, and for assessing therapeutic success. MRI techniques have improved over the last few decades, revealing not just morphologic information, but functional information about perfusion, diffusion and hemodynamics as well. Four-dimensional (4D) flow MRI, a time-resolved phase contrast-MRI with three-dimensional (3D) anatomic coverage and velocity encoding along all three flow directions has been used to comprehensively assess complex cardiovascular hemodynamics in multiple regions of the body. The technique enables visualization of 3D blood flow patterns and retrospective quantification of blood flow parameters in a region of interest. Over the last few years, 4D flow MRI has been increasingly performed in the abdominal region. By applying different acceleration techniques, taking 4D flow MRI measurements has dropped to a reasonable scanning time of 8 to 12 min. These new developments have encouraged a growing number of patient studies in the literature validating the technique's potential for enhanced evaluation of blood flow parameters within the liver's complex vascular system. The purpose of this review article is to broaden our understanding of 4D flow MRI for the assessment of liver hemodynamics by providing insights into acquisition, data analysis, visualization and quantification. Furthermore, in this article we highlight its development, focussing on the clinical application of the technique.

Key words: Four-dimensional flow magnetic resonance imaging; Phase contrast-magnetic resonance imaging; Liver cirrhosis; Imaging technique; Hemodynamics; Blood flow; Visualization; Quantification; TIPS; Splanchnic system

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Core tip: Liver cirrhosis is one of the leading cause

of morbidity and mortality in the United States, Europe and Asia. Advanced stages of liver cirrhosis are accompanied by hemodynamic changes of the hepatic vascular system. Four-dimensional (4D) flow magnetic resonance imaging (MRI) has been validated for the clinical assessment of the liver blood flow in patients with advanced liver cirrhosis. It represents a method that supplements Doppler ultrasound and provides important additional information on the vessel system in difficult patients. The purpose of this review is to provide insights into 4D flow MRI for blood flow visualization and quantification in patients with advanced liver cirrhosis.

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INTRODUCTION

Liver cirrhosis is a leading cause of serious morbidity and the 9th most often cause of death in the United States and Europe with a mortality of more than 35000 deaths per year[1,2]. The most common causes of liver cirrhosis in developed countries are alcoholic liver disease and hepatitis C, while in most parts of the sub-African continent and in Asia, hepatitis B dominates as the predominant cause[3-5]. Liver cirrhosis reflects the histological development of regenerative nodules and fibrotic bands as a response to chronic liver injury. In the cascade of liver damage, it appears at advanced stages of liver fibrosis and is accompanied by distortion of the hepatic vascular system. The hemodynamic changes in the liver result in the shunting of the portal venous and arterial blood supply directly into the central hepatic veins, bypassing the exchange between hepatic sinusoids and the adjacent hepatocytes[6,7]. The major clinical effects of the liver damage and liver cirrhosis are impaired liver function, portal hypertension with increased intrahepatic resistance, and potential development of malignant hepatocellular carcinoma. The hemodynamic changes in liver cirrhosis are closely associated with portal hypertension and vascular alterations in the liver parenchyma[8,9].

Over the last 3 decades, phase contrast (PC) MRI has become established in the clinical routine for diagnosing hemodynamic alterations in the heart, aorta and large thoracic and abdominal vessel systems[10-12]. Further improvements in PC-MRI led to time-resolved (CINE) 3-dimensional (3D) PC-MRI technique with the feasibility of three-directional velocity encoding [four-dimensional (4D) flow MRI]. 4D flow MRI enables 3D volumetric coverage and the means of assessing hemodynamic changes in a specific region over time[13-17].

The purpose of this review article is to provide insights into the 4D flow MRI imaging technique for blood flow visualization and flow quantification in patients with advanced liver cirrhosis and in healthy volunteers. I also aim to describe the clinical advantages and disadvantages of the method, illustrating recent developments of the technique in the liver's vascular system as well as presenting several very recent clinical applications of the method for evaluating hemodynamic anomalies.

DIAGNOSTIC MODALITIES FOR LIVER FIBROSIS AND LIVER CIRRHOSIS

A perfect non-invasive test for patients with liver cirrhosis would be simple to perform, safe, inexpensive, accurate, reproducible, yield numeric results in real time, reflect therapy-induced changes, enable prognostic stratification, and predict potential long-term outcomes related to liver cirrhosis[18-20].

Liver biopsy is acknowledged as the gold standard when diagnosing liver cirrhosis and assessing the stage and grade of chronic hepatitis[21-24]. The histological sub-classification and quantitative evaluation of hepatic fibrosis reveals a correlation with the disease's clinical stage and prognosis, and is valuable in validating other non-invasive markers[25,26]. The repeatability of liver biopsy and its clinical acceptance by the patients is limited by its potential for complications, sampling errors, inter- and intra-observer variability, and invasiveness[27,28]. In patients with liver cirrhosis, measurements of the hepatic venous pressure gradient (HVPG) provide an accurate estimate of portal pressure and offer a solid clinical and prognostic marker for chronic liver disease[29-32]. It enables us to assess the development of clinical complications, *e.g.* esophageal varices and bleeding and the risk of decompensation, and provides independent prognostic information on patients' survival and future mortality risk[33-37]. Similar to liver biopsy, HVPG's main limitation is its invasiveness and limited availability[32]. Much effort has been made to identify a non-invasive alternative offering comparable reliability to the invasive interventions described above.

An important clinical role is played by laboratory tests such as biochemical and hematological serum markers with the benefits of simple, non-invasive and repetitive testing and results reflecting the entire liver. Serum markers are "indirect" and "direct": the "indirect markers" reveal the degree and stage of fibrosis, while "direct markers" indicate the enzymes playing a key role in matrix regulation or the hepatic matrix components and their deposition or removal[38,39]. Studies evaluating serum markers have demonstrated their predictive efficiency for fibrosis and cirrhosis[40,41] although a meta-analysis describes their accuracy as limited in assessing the fibrosis stage[42]. To optimize reliability, a combination of serum markers is used instead of a single marker[43]. Nevertheless, non-

invasive assessment of the dynamic changes in portal hypertension, liver fibrosis and cirrhosis by laboratory serum markers remains inadequate[42,43].

Diagnostic imaging modalities have a fundamental function in managing patients with chronic liver disease and in diagnosing the malignant transformation to hepatocellular carcinoma. The clinical gold standard and most comprehensive available and evaluated imaging test is ultrasound (US)[44-48] and accompanied techniques including transient elastography (TE)[49,50], acoustic radiation force impulse (ARFI)[51] and supersonic shear-wave elastography (SSWE)[52] imaging. Based on its broad availability, non-invasive character and easy applicability, Doppler US is well established in the clinical routine for the follow-up and assessment of patients with chronic liver disease and liver cirrhosis[44-48]. In advanced stages of liver fibrosis, morphological alterations are accompanied by changes in the liver's blood flow[53]. Assessment by US include liver and spleen size measurements, liver parenchyma evaluation, but also measurements of portal vein (PV) velocities, PV blood flow, liver perfusion, and resistance indices in splanchnic arteries[53-57]. Ultrasound is reported to demonstrate very low sensitivity and specificity for liver fibrosis, and no correlation was detected between US results and liver biopsies when staging liver fibrosis[58,59]. Better assessment of hemodynamic changes in the liver has been made possible by contrast-enhanced ultrasound (CEUS)[60,61]. There is evidence that regional hepatic perfusion parameters correlate with the severity of liver failure and are increased in patients with liver cirrhosis[61]. The usual US limitations are involved in this technique, as the involvement of different contrast agents with different kinetics and drug design, varying operator skills and inconsistent availability of the method. Other limitations are the patient-related and operator-dependent conditions. US is influenced by the stage of NPO (nothing per mouth), patient respiration, ascites, bowel gas, differences in equipment and inter-observer variability in the measurements[62-66].

Other ultrasound-based techniques focus on the mechanical properties of liver tissue and measure differences in viscoelasticity in patients with liver fibrosis[51]. The modalities in evaluating liver stiffness in hepatic fibrosis are useful to reduce invasive pressure measurements, predict lethal complications or improve patient's prognoses and risk stratification. The two most frequent modalities are either real-time based elastography or shear-wave elastography. TE is the most widely applied and tested modality, followed by ARFI[51] and SSWE[52]. Numerous studies have shown that TE results correlate significantly with the histological stage of liver fibrosis and are very diagnostically accurate[67-69]. Nevertheless, TE has a high measurement failure rate of up to 20% due to limitations like severe obesity, ascites and subcutaneous fat[49,60,70]. Further restrictions for its broad clinical use are its wide range of cut-off values and results variability across different studies[71-74].

Conventional CT and MRI are well-suited for evaluating morphological anomalies in patients with chronic liver disease, as they show the degree of liver injury from cirrhosis and accompanying complications. Alterations result from portal hypertension, hepatic insufficiency and portosystemic shunting, which result in ascites, gastrointestinal bleeding, coagulopathy, encephalopathy, and the formation of collateral vessels and portosystemic shunts[75-77]. Predisposing locations of the collateral vessels are distal esophageal, the gastric fundus, paraumbilical, splenorenal, retroperitoneal, abdominal wall and hemorrhoidal[78,79]. These morphologic changes in liver hemodynamics are usually visible in advanced stages of liver cirrhosis. As a result, CT and MRI are unsuitable for diagnosis of the early stages of liver cirrhosis, including functional evaluation of the liver blood flow. An MRI-based assessment of liver stiffness by MR elastography (MRE) offers better contrast between different body tissues than Ultrasound[80-82]. Further advantages of the technique is its potential to assess the whole liver, observer-independence, and no influence by the body habitus[83,84]. MRE has become standard for assessing liver fibrosis as it offers generally high sensitivity and specificity for different histological gradings[81-86]. Factors associated with this technique that limit its use in the daily clinical routine are its expense and restricted availability[83,84].

Much experience has been gained over the last two decades in using MRI to hemodynamically assess the liver blood flow[87-90]. Most published studies relied on 2D PC-MRI measurements, a robust and reliable technique for hemodynamic assessment of the liver. It has revealed low intra- and inter-observer variability and high reproducibility compared to Doppler US[87-90]. 2D PC-MRI is, however, limited by the application and positioning of 2D planes. Flow measurements and flow quantification results obtained *via* 2D PC-MRI can be unreliable because of difficulties in precise orthogonal positioning of the measurement plane within the complex liver vascular system[91]. Another large group of studies assessing liver hemodynamics relied on contrast-enhanced MRI. Since nephrogenic systemic fibrosis (NSF) has been observed with its potential connection to the injection of gadolinium-based contrast medium, contrast medium is being more carefully applied[92-96].

Until now, no non-invasive diagnostic modality has been able to determine the changes in and exact stage of chronic liver disease and portal hypertension. Nevertheless, new modalities reveal promising results in hemodynamic assessments of the liver. Further clinical trials examining different disease stages are needed to validate the reproducibility and long-term prognostic values of these non-invasive diagnostic modalities. These tests have the potential to lead to unknown and new paradigms in the specific management of patients with chronic liver disease.

4D FLOW MRI

During data acquisition with 4D flow MRI, velocity is encoded along all three spatial directions throughout the entire heart cycle. This results in a time-resolved volumetric velocity field from the scanned body region, *e.g.* heart, aorta, lung, intracranial vessels or liver[10,97,98]. To take quantitative velocity measurements in one spatial direction, two data acquisitions and the following subtraction are needed for velocity encoding. Seeking velocity encoding in all three spatial directions, one needs a reference image and three velocity-encoded images acquired along the three orthogonal directions X, Y and Z[99-101]. The data is acquired over several cardiac cycles, while data acquisition at the same time is synchronized with the heart cycle using the k-space segmentation technique. The 4D flow MRI data is thus measured only partially during one heart cycle; the entire data is acquired ongoing over several heart cycles. After completing 4D flow MRI data acquisition, 4 time-resolved 3D flow datasets are generated: one dataset with the magnitude information containing anatomic information and three flow datasets giving the velocities in each spatial direction X, Y and Z. The extended amount of data encoding for three spatial dimensions, three velocity directions, and the time information over the cardiac cycle is reflected in the scan time. Several recent examinations in 4D flow MRI sequencing addressed this limitation and tried to find a potential solution to shorten the scan time. While the manufacturers continuously improve the hardware aspect of the scanners, others are working on software improvements.

Fast imaging techniques like radial imaging 3D PC-VIPR (vastly undersampled isotropic projection reconstruction)[102,103], spiral techniques without acceleration[104], compressed sensing[59,105] or parallel imaging enable 4D flow MRI scans within 8 to 20 min[15,106]. Conventional acceleration techniques like GRAPPA (generalized auto-calibrating partially parallel acquisitions)[57] or SENSE (sensitivity encoding)[55] can usually achieve a scan time reduction by factor 2 or 3. Applying higher values could negatively influence the quantification of velocities with a reduced signal-to-noise ratio[107]. Further advancements in spatial-temporal parallel imaging techniques such as k-t PCA (k-t principal component analysis)[108], k-t GRAPPA[109-111], k-t SENSE[112,113] or k-t BLAST[113,114] facilitate higher acceleration factors and represent promising imaging techniques for scan time reduction using 4D flow MRI. Several studies have already used a radial data acquisition technique combined with under sampling of the data (PC-VIPR) for assessing the arterial and portal venous system within the liver and splanchnic vessels[115-117]. This imaging technique offered shorter scan times together with broad volumetric coverage and enhanced spatial resolution, fewer motion artifacts and self-gating. A recent study evaluated scan time savings using a k-t GRAPPA accelerated Cartesian 4D flow MRI to visualize and quantify liver hemodynamics[118]. Three different acceleration factors were used, with additional focus on temporal resolution. All k-t GRAPPA acceleration factors displayed significant scan time savings, R = 3 and R = 5 over 40% to almost 8 min and R = 8 over 70% with a 4D flow MRI scan time of almost 4 min. While acceleration factors R = 3 and R = 5 showed quantitative blood flow and velocity results comparable to standard GRAPPA R = 2, acceleration factor R = 8 revealed increased noise and artifacts with significantly lower measurement results in the arterial system according to the calculated blood flow parameters[118]. Another current study is evaluating a combined-spiral-sampling and dynamic-compressed-sensing approach for faster acquisition of 4D flow MRI[119]. In a study cohort with 10 subjects, investigators have demonstrated the feasibility of applying 4D flow MRI within an average scan time of 24 s (18-25 s range), comparable to 2D PC-MRI measurements. Moderate to substantial agreement was observed in the delineation of arterial and venous vessel borders between the spiral 4D flow MRI and the Cartesian 4D flow MRI approach. Quantitative results revealed good agreement and a 95% confidence interval between 60% and 77% for the flow parameters acquired[119]. These recent studies addressing the acceleration of 4D flow MRI for abdominal imaging show the potential for this technique to be accelerated to last a few seconds while enabling comprehensive evaluation of liver hemodynamics. For thoracic or abdominal applications of the 4D flow MRI technique, breathing control and ECG-gating is needed to reduce consequent artifacts. In addition to breathing bellows or navigator gating, self-gating methods have been reported[120-123]. Upcoming studies will continue to validate the scan-time savings results using various acceleration techniques and offering broad clinical application for patients with advanced liver cirrhosis as well as better understanding of complex blood flow changes in the liver.

Technical aspects for data analysis

Many technical aspects must be considered when performing 4D flow MRI data acquisition and validating the data acquired. A detailed description and discussion of these technical aspects is beyond the scope of this clinically-focused review article, and I offer only an overview of the most important facts. For further detailed information, please consult comprehensive review articles for 4D flow MRI, *e.g.*[16,17].

Velocity encoding sensitivity (Venc) represents the peak flow velocity that can be acquired. If the peak velocity of the blood flow in the vessels exceeds the preset setting for the Venc, the accuracy of flow visualization and quantification of the liver

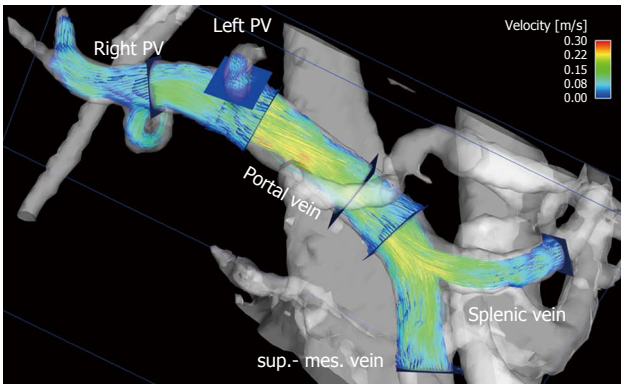

Figure 1 Particle traces visualization of portal venous flow in a healthy volunteer. Blue emitter planes were positioned in the splenic vein and superior mesenteric vein (sup.-mes. vein), portal vein, right (right PV) and left (left PV) intrahepatic portal vein branch. The gray-shaded iso-surface three-dimensional angiogram has been calculated from the 4D flow magnetic resonance imaging data. Color-coded peak velocities in the inflow of the portal vein from the splenic and superior mesenteric veins and the intrahepatic part of the portal vein.

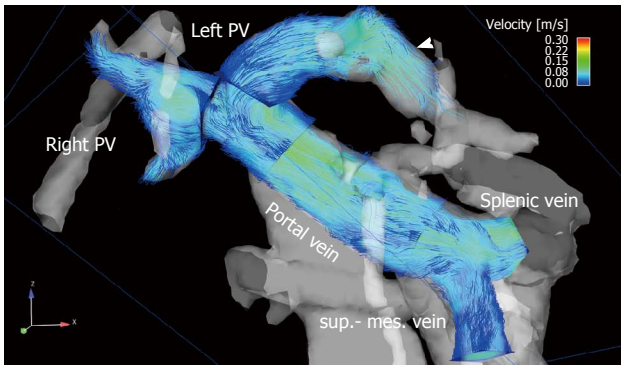

Figure 2 The portal venous system visualized by particle traces in a 68-year-old male patient with liver cirrhosis (Child-Pugh stage B). Blue emitter planes were positioned in the splenic vein and superior mesenteric vein (sup.-mes. vein), portal vein, right (right PV) and left (left PV) intrahepatic portal vein branch. Time-resolved particle traces illustrate physiological flow in the extrahepatic portal venous system with inflow in the portal vein from the splenic and superior mesenteric veins. Flow over the left branch of the intrahepatic portal vein into a re-opened umbilical vein is visible (arrowhead).

hemodynamics may be compromised and anti-aliasing corrections are necessary[124]. To obtain optimal image quality, the Venc should represent the hemodynamic conditions in the hepatic area and be as high as necessary to avoid anti-aliasing, but as low as possible to reduce velocity noise. A typical Venc setting for the portal venous system is 50 cm/s, for the arteries 100 cm/s, and values even above 150 cm/s within a TIPS stent[16,17]. An interesting approach for the liver with different flow velocities is a dual-venc, already discussed in other regions of the body[125].

Many errors in 4D flow MRI can impair image quality and trigger inaccuracies in quantitative flow measurements. The most common errors are phase offset errors based on gradient field nonlinearity[106], eddy currents[126] and Maxwell terms[127]. Before further processing the 4D flow MRI data, it is important to

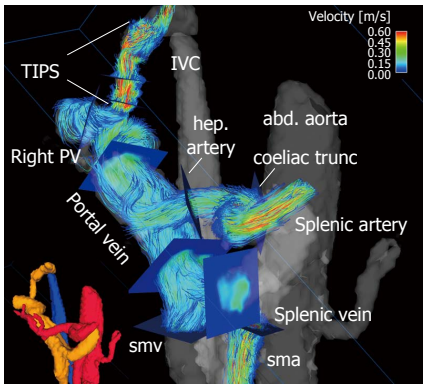

Figure 3 Four-dimensional flow magnetic resonance imaging in a 63-yr-old female with liver cirrhosis after TIPS placement. Small lower left corner: three-dimensional (3D) segmentation of the liver and splanchnic hemodynamics (red: arterial system; orange: portal venous system; blue: venous system). Large figure: Color-coded 3D particle-traces visualization demonstrates increased velocities within the TIPS and the arteries. Blue analysis planes were positioned throughout the arterial and portal venous systems as well as TIPS to quantify liver hemodynamics. abd. aorta: Abdominal aorta; hep. artery: Hepatic artery; sma: Superior mesenteric artery; smv: Superior mesenteric vein; Right PV: Right portal vein branch; IVC: Inferior vena cava.

take such errors into account and consider appropriate correction strategies.

To visualize the 4D flow MRI data acquired, many options are available on the market, usually using 2D analysis planes which must be positioned in the vessel of interest[11,128-133] (Figures 1 and 2). Time-resolved particle traces and 3D streamlines are emitted from these analysis planes. Time-resolved particle traces display the temporal evolution of blood flow over one or more cardiac cycles[132]. Velocity changes can be visualized or the flow pattern traced to its origin by color-coding the particle-traces. Streamlines represent 3D traces that track the spatial distribution of 3D velocities within an individual cardia time frame. Color-coding the streamlines enables visualization of the spatial distribution and orientation, especially of peak blood flow velocities.

4D flow MRI has the potential to retrospectively quantify hemodynamic parameters of the liver at any location within the 3D data volume following data acquisition[134,135]. 2D analysis planes can be freely positioned in the interesting arterial or portal venous vessel to quantify standard flow parameters like peak and mean velocities, flow volume over the cardiac cycle, vessel area, shunt fraction or flow reversal (Figures 2 and 3). Several studies report excellent agreement between 2D PC-MRI and 4D flow MRI in flow quantifications[136,137]. Good scan-rescan reproducibility and low inter- and intra-observer variability have been shown in conjunction with 4D flow MRI flow quantification in the intracranial, cervical, thoracic and abdominal vessel systems[136,138,139]. New strategies are also discussed in the literature for evaluating more advanced hemodynamic parameters, *e.g.* wall shear stress, pressure difference, turbulent

kinetic energy and pulse wave velocity[135,140-143].

Blood flow visualization

4D flow MRI represents a non-invasive and observer-independent technique for comprehensive 3D volumetric evaluation of the liver and splanchnic region, as well as the means of assessing the liver's arterial and portal venous system, collateral vessels, and potential shunts. In addition to initial studies evaluating flow in the portal vein[89,144-146] the focus of the first feasibility studies using 4D flow MRI in the liver was on the entire portal venous system[147,148]. These studies addressed the inflow from the splenic and superior mesenteric vein, the splenic-mesenteric confluence, the intrahepatic portal vein and right and left intrahepatic branches[147,148]. Initial studies comparing healthy volunteers and patients with liver cirrhosis applied a velocity encoding of 50 cm/s and attained complete visualization of the extrahepatic vessels in over 94% of their subjects (Figure 2). The small intrahepatic branches presented a limitation, however, as complete visualization was only possible in about 80% of the subjects[147,148]. Another group applied radial 4D flow MRI acquisition[102,103] and demonstrated good to excellent visualization in patients with liver cirrhosis, identifying all arterial vessels and 86% of the portal venous circulation[115,116]. Subsequent studies using Cartesian 4D flow MRI and a velocity encoding of 100 cm/s were also able to evaluate the liver's arterial and portal venous system[118,149]. Complete visualization of the arterial system was accomplished in liver cirrhosis patients and volunteers in almost 100% of the cases. The limiting factor in the volunteer studies was the left intrahepatic branches (complete visualization in 50% to 60% of the cases)[118,149]. In the patient cohort studies the limitations in the portal venous system were more obvious as complete visualization of the extrahepatic vessels was only possible in in 60% to 90% of the cases, while the small intrahepatic branches of the portal vein were completely visible in only 20% to 60% of the cases[150,151]. A possible reason for these limitations might be in the spatial resolution of the 4D flow MRI sequence and the quite long scan time under free breathing during acquisition, resulting in partial volume effects and signal blurring due to organ motion and reduced effective spatial resolution in the Z-direction. In addition to visualization of the hepatic hemodynamic system, a major advantage of the 4D flow MRI sequence is its ability to evaluate the blood flow direction. Qualitative results in a feasibility study using radial 4D flow MRI depicted reverse hepatofugal flow in 5% of the portal venous vessels[115]. Using Cartesian 4D flow MRI, that study group showed a reopened umbilical vein in 6 out of 20 liver cirrhosis patients with portosystemic shunts[148]. In another patient-cohort study addressing more advanced stages of liver cirrhosis portosystemic collateral vessel systems were visualized prior to TIPS revision in 8 out of 11 cases[151]. The shunt was not visible in the follow-up 4D flow MRI examination after insertion of a TIPS stent graft[151,152]. The successful occlusion of the shunt was proven during the intervention. The results of these studies also highlight one of the limitations of the 4D flow MRI sequence. Due to the 100 cm/s velocity encoding favoring high blood flow velocities and the applied spatial resolution, it can be difficult to distinguish the collateral vessels having a small diameter and low blood flow velocities in advanced stages of the liver disease. Precise differentiation of the regressive development of collateral vessels might be difficult when referring to 4D flow MRI using only the actual sequence parameter. Better spatial resolution would improve visualization of the portal venous vessel system and the collateral vessels with low blood flow velocities and small vessel size in advanced stages of liver disease. A further potential limiting factor is the signal-to-noise ratio. The above-mentioned studies involving radial acquisition applied contrast medium, while the Cartesian-based studies did not. The influence of the contrast medium, field strength and better respiratory triggering on the 4D flow MRI for the liver hemodynamics should be examined in future studies with larger patient cohorts.

Retrospective blood flow quantification

As well as visualizing 3D blood flow characteristics and clinically illustrating complex alterations in patients with advanced liver disease, 4D flow MRI enables the comprehensive quantification of blood flow parameters from the same dataset. Several working groups have conducted a quantitative evaluation of flow parameters in the portal venous system using Doppler US or MRI, while most of the MRI results are based on 2D PC-MRI measurements[144,145,153-156]. Initial studies using 4D flow MRI within the portal venous system displayed moderate, but significant correlations among 4D flow MRI, 2D PC-MRI and Doppler US values[147,148]. The peak-velocity results were between 23-27 cm/s, slightly lower than in other studies reporting 28 cm/s using 2D PC-MRI and Doppler US[89]. Mean velocity values between 10-12 cm/s resemble those in other studies based on 2D PC-MRI (11-14 cm/s)[144,145,154]. However, published values from studies applying Doppler US reveal higher mean velocities between 15-17 cm/s[156,157]. The peak and mean velocities of blood flow in the liver tended to be underestimated in conjunction with 4D flow MRI (between 35% and 38%)[147,148]. Another 4D flow MRI study evaluating blood flow in the carotid bifurcation offered a similar underestimation of flow velocities (between 31% and 39%) *via* 4D flow MRI compared to Doppler US[158]. An explanation for MRI's tendency to underestimate velocities is related to the data acquisition method. The velocity data is acquired over several cardiac cycles, resulting in an average velocity progression. Velocity

changes within the different heart cycles and short time fluctuations are not displayed. In comparison: Doppler ultrasound measurements represent real-time velocity data[144,156]. MRI's underestimation of velocities therefore has to do with velocity averaging. Another reason might be partial volume effects due to lower spatial resolution in MRI than in Doppler US.

A 4D flow MRI study detected lower flow-volume values after measuring mean velocities and vessel area (mean 0.7 ± 0.4 L/min)[148] compared to 2D PC-MRI studies (between 1.0 and 1.3 L/min)[89,145,155]. Doppler US studies, however, yielded a wide range of flow volumes (between 0.3 and 1.3 L/min)[159,160]. One reason for these different flow volumes could be hemodynamic changes after ingestion. A Doppler US-based study evaluating postprandial hyperemia in patients with liver cirrhosis revealed an average increase in portal venous flow velocities of 29% and a 38% increase in the portal venous blood flow[161]. Most recent studies evaluating reproducibility and the postprandial effect *via* 4D flow MRI report good to excellent short-term reproducibility in the fasting state[162,163]. Portal venous flow parameters were significantly higher in the postprandial state, confirming the large impact of caloric intake on portal venous flow[162]. Portal venous flow regulation might also be impaired after a meal challenge in patients with liver cirrhosis[163].

Recent 4D flow MRI studies measuring the vessel area report higher values compared to Doppler US in correlation to earlier studies with 2D PC-MRI and Doppler US[144,145,147,148,164]. One reason for the differences between these two modalities might be the location and angle of the ultrasound transducer during vessel diameter assessment, which would compromise reliable measurements not just in small vessels. The differences are inter-observer and intra-observer variability[157].

A recent reproducibility study examined the reliability of 4D flow MRI data acquired from the thoracic aorta with high flow velocities[138] showing good scan-rescan reliability and low inter- and intra-observer variability in the acquired and clinically-relevant blood-flow parameters and in wall shear stress (WSS)[138]. *Via* radial acquisition, another study group confirmed the results from quantifying hepatic and splanchnic hemodynamics using 4D flow MRI[116]. Investigating patients with portal hypertension, that study revealed good internal data consistency and low inter- and intra-observer variability in 4D flow MRI data from the liver's vascular system[116]. They validated their results indirectly by being internally consistent at three different locations within the vascular system. Taking measurements at three different locations in the portal vein revealed an average absolute error of 4.2% ± 3.9%. Comparison of flow into the portal confluence coming from the splenic and superior mesenteric veins with the flow in the portal vein yielded an error of 5.9% ± 2.5%. Assessment of flow in the portal bifurcation and the right and left intrahepatic portal vein branches showed an error of 5.8% ± 3.1%[116]. Another study applying Cartesian 4D flow MRI revealed similarly good results with small errors in the internal consistency validation of the flow parameters[150]. Those authors performed additionally a real scan-rescan validation of 4D flow MRI with a rescan of all volunteers at least 5 mo later on the same scanner. They reported good reproducibility of 4D flow MRI quantification in the portal venous system with low blood flow velocities offering a mean average difference of 2% between the two scans for peak velocities and 5% for the mean velocities[150]. Quantitative evaluation of arterial blood flow velocities using 4D flow MRI yielded robust results with mean average differences of 3% for peak velocities and 7% for mean velocities[150]. Reproducibility of flow volume assessments showed low error for mean average differences of 6% in the portal vein, while the arterial flow volume evaluation was limited by an error of 14%. Inter-observer variability of between 16% and 26% is described in the literature in association with the evaluation of portal venous blood flow velocities using the clinical gold standard, Doppler US[165]. 4D flow MRI has yielded similar results: a scan-rescan-variability between 25% and 26% in the assessment of flow velocities[150]. A possible reason for these variations is the manual segmentation of vessel borders. A semi- or full-automatic segmentation method could improve the reproducibility of such calculated parameters and those derived from the vessel area[166].

Doppler US-based studies describe significantly low flow velocities and flow volumes in patients with advanced liver cirrhosis compared to healthy volunteers[159,167]. In a study taking the radial 4D flow MRI approach, the MELD score was calculated to estimate disease severity and correlated with image quality[115], yielding no correlation between image quality and the MELD score. Another study analyzed their patient cohort applying the Child-Pugh score to assess the degree of liver failure compared to the quantitative flow parameters derived from 4D flow MRI data and Doppler US measurements[148]. They detected no relevant correlation between disease severity and changes in liver hemodynamics; the expected changes were only visible in few hepatic vessels. Those 4D flow MRI findings might be associated with the study patients they recruited. In both 4D flow MRI studies, most of the cohorts' liver cirrhosis patients presented an early stage of the disease and a low MELD score or Child-Pugh stage A with subsequently few anomalies in liver hemodynamics[115,148]. The Doppler US studies reveal upon closer inspection to have included patients with mainly advanced liver cirrhosis (Child-Pugh stage C) and more advanced impairments in the hepatic vessel system[159,167]. To further validate 4D flow MRI data, additional patient studies are needed with larger patient cohorts and involving different stages of disease severity.

The most interesting novelty represented by 4D flow MRI is that it allows us to reliably assess both the portal venous system in patients with altered liver hemodynamics, as well as small arteries in the liver and splanchnic system[115,116,118,149,151] (Figure 3). It is a technique that offers quantitative equilibrium in the patient's blood flow between the arterial inflow to the liver and splanchnic system and the portal venous outflow to the liver parenchyma. We can thus calculate the shunt fraction in patients with advanced liver cirrhosis and a portosystemic shunt[151,152]. An increase in liver perfusion of 424 mL/min can be verified after TIPS intervention by assessing the different flow rates in the hepatic arteries, portal vein and TIPS stent-graft[151]. Doppler US studies reveal an increase in flow velocities after a TIPS intervention by a factor of 2 to 4[168,169]. 4D flow MRI data has demonstrated results similar to Doppler US's in peak velocities in the portal vein before TIPS insertion (4D flow MRI: 19 ± 5 cm/s *vs* Doppler US 10-20 cm/s), but revealed lower values in the portal vein during TIPS follow-up (4D flow MRI: 28 ± 7 cm/s *vs* Doppler US 40-60 cm/s)[151,169]. Normally-functioning stent-shunts yielded in-stent values *via* 4D flow MRI comparable to those in Doppler US with peak velocities measuring between 50 cm/s and 200 cm/s (range 58-194 cm/s)[151,169]. Stenoses within the stents were reliably depicted and confirmed by invasive catheter pressure gradient measurements during stent shunt revision[151]. These recent studies evaluating 4D flow MRI for abdominal imaging after TIPS placement show the potential for this technique to be an additional tool for interventional radiologists while enabling pre-procedure mapping and planning of the optimal stent graft configurations[151,152]. As a result, ideal outcome after TIPS placement can be obtained including pressure gradient reduction and long-term stent graft patency.

DISCUSSION

There is ample evidence that 4D flow MRI has potential to image the hemodynamics in patients with advanced liver cirrhosis and to measure altered blood flow parameters.

One of the main limitations of 4D flow MRI investigations addressing the liver and visceral blood flow is the small size of the patient and control cohorts in the clinical studies. This has a lot to do with the advanced clinical stages of patients suffering from worsening liver cirrhosis. It is difficult to obtain accurate data in patients with severe ascites, moreover, the compliance of patients with advanced stages of the disease is often poor. The imaging potential and therapeutic tools are sometime only palliative when treating patients with advanced liver cirrhosis, and we often have access to too few patients (*e.g.* Child-Pugh stage C). Their high mortality rate also makes a longitudinal study design with longer follow-up controls more difficult than, for example, examining patients with heart diseases. Nevertheless, our aim should be to carry out large multicenter cohort studies reflecting different manifestations of liver cirrhosis in order to further validate the 4D flow MRI technique in a clinical setting.

Validation of the 4D flow MRI method is another limitation: studies have already compared 4D flow MRI to Doppler US, the clinical gold standard, in many body regions[158,170] including hepatic and visceral blood flow[116,149]. 4D flow MRI provides good scan-rescan variability and low inter- intra-observer variability[116,149,158,170], although most of those studies' subjects were healthy volunteers, and few patients with liver cirrhosis were monitored during follow-up. We will need further clinical cohort studies to assess the accuracy of the quantitative 4D flow MRI results in comparison with invasive measurements of hemodynamic parameters.

A further limiting factor of the 4D flow MRI sequence is still the spatial and temporal resolution. The Cartesian 4D flow MRI approach has particular limitations in capturing the small intrahepatic vessels and hepatic arteries. With its superior resolution, the radial 4D flow MRI sequence visualizes the vessel system impressively. 4D flow MRI methods require further improvements to enhance the accuracy of quantitative results in the small vessels. Another limitation of 4D flow MRI compared to Doppler US is the acquisition of data over several cardiac cycles averaging over time. This issue is a concern in the arteries especially, less so in the portal venous system, whose peak velocities and flow volume values are slightly lower and do not reveal brief time variations in blood flow. The 4D flow MRI sequence is still being researched. The shortage of freely-available software packages, the time it takes to perform the pre- and post-processing, and the lack of a standardized approach for data evaluation are additional factors that limit the broader clinical application of this method. A collective endeavor is needed from clinicians, researchers and manufacturers to pave the way for greater availability of the 4D flow MRI sequence and consequent increased clinical applications. Further developments should focus on refining the clinical workflow, on presenting the acquired data to clinical colleagues, and on improving the accessibility of results from within existing patient archives.

CONCLUSION

4D flow MRI has been validated for the clinical assessment of the liver blood flow in patients with advanced liver cirrhosis. It is an MRI technique that can examine the patient from a functional perspective as a part of "one-stop-shopping". More importantly, 4D flow MRI is a method that supplements Doppler US. It provides important additional information on the vessel system in difficult patients. The potential of 4D flow MRI is growing; the more advanced it becomes, the better we will understand the pathophysiology of liver

cirrhosis and the dynamic alterations it causes. That, in turn, will ensure better patient management and more accurate risk stratification.

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TOPIC HIGHLIGHT

2016 Cirrhosis: Global view

Magnetic resonance imaging of the cirrhotic liver in the era of gadoxetic acid

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Abstract

Gadoxetic acid improves detection and characterization of focal liver lesions in cirrhotic patients and can estimate liver function in patients undergoing liver resection. The purpose of this article is to describe the optimal gadoxetic acid study protocol for the liver, the unique characteristics of gadoxetic acid, the differences between gadoxetic acid and extra-cellular gadolium chelates, and the differences in phases of enhancement between cirrhotic and normal liver using gadoxetic acid. We also discuss how to obtain and recognize an adequate hepatobiliary phase.

Key words: Hepatobiliary contrast materials; Gadoxetic acid; Cirrhosis; Magnetic resonance imaging; Liver

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Core tip: Hepatobiliary contrast materials improve detection and characterization of focal liver lesions in cirrhotic patients and can measure liver function. Familiarity with unique characteristics of gadoxetic acid is crucial to achieve an optimal magnetic resonance examination of the liver. In this review, we discuss the protocol for gadoxetic acid enhanced magnetic resonance imaging of the liver and describe differences

between gadoxetic acid and extra-cellular contrast materials.

Agnello F, Dioguardi Burgio M, Picone D, Vernuccio F, Cabibbo G, Giannitrapani L, Taibbi A, Agrusa A, Bartolotta TV, Galia M, Lagalla R, Midiri M, Brancatelli G. Magnetic resonance imaging of the cirrhotic liver in the era of gadoxetic acid. *World J Gastroenterol* 2016; 22(1): 103-111 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i1/103.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i1.103

INTRODUCTION

Several studies have demonstrated the added value of hepatobiliary contrast agents in the detection and characterization of focal liver lesions in cirrhotic patients compared with extra-cellular gadolinium chelates and contrast enhanced computed tomography (CT)[1-4]. Hepatobiliary contrast agents are first distributed in the extracellular fluid compartment, subsequently taken up by functioning hepatocytes, and then excreted into the biliary system[5,6]. Thus, hepatobiliary contrast agents can differentiate lesions that contain functioning hepatocytes, such as regenerative nodules and most dysplastic nodules, from hepatocellular lesions without functioning hepatocytes, such as most hepatocellular carcinomas (HCCs) and nonhepatocellular lesions, such as cyst, hemangioma, cholangiocarcinoma, metastases[7].

There are two commercially available hepatobiliary contrast agents: gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (gadoxetic acid; Eovist/Primovist; Bayer-Healthcare, Leverkusen, Germany) and gadobenate dimeglumine (Multihance, Bracco, Italy). Both of them allow evaluation of lesion vascularity and hepatobiliary function. However, approximately 50% of the injected dose of gadoxetic acid is eliminated by functioning hepatocytes, while only 3%-5% gadobenate dimeglumine undergoes the same pathway of excretion[5,6]. Therefore, using gadoxetic acid, higher hepatobiliary uptake results in greater enhancement of liver parenchyma[8].

Another unique feature of gadoxetic acid is the rapid hepatocellular uptake (starting at approximately 90 s after injection)[1], which results in an overlap between extracellular and hepatobiliary phases (the so-called "transitional phase"). Rapid uptake of gadoxetic acid allows acquisition of the hepatobiliary phase at 20 min after contrast injection[1]. Hepatocellular uptake of gadobenate dimeglumine starts no sooner than 40 min after contrast injection[5]. Therefore, the extracellular phase of gadobenate dimeglumine is "pure" (it shows no overlap with the hepatobiliary phase, similar to what can be obtained with any extracellular contrast agent), and the hepatobiliary phase is typically acquired 60-180 min after contrast injection[9]. Thus, with gadobenate dimeglumine, dynamic and hepatobiliary images are acquired in two separate sessions, increasing examination time and patient discomfort. For these reasons, gadoxetic acid is generally preferred over gadobenate dimeglumine when acquisition of hepatobiliary phase is deemed necessary for the management of patients. The main disadvantage of liver magnetic resonance imaging (MRI) with gadoxetic acid is the contrast cost: the purchase price of gadoxetic acid is approximately twice that of gadobenate dimeglumine. As MRI reimbursements in the public sector are fixed, many institutions use gadobenate dimeglumine instead of gadoxetic acid for economic reasons.

In this review, we describe the optimal MRI study protocol of the liver and the differences in phases of enhancement between cirrhotic and normal liver using gadoxetic acid. We also illustrate the differences in phases of enhancement between gadoxetic acid and extracellular contrast agents and discuss how to obtain and recognize an adequate hepatobiliary phase.

WHY GADOXETIC ACID IN THE CIRRHOTIC LIVER

The need for an accurate detection and characterization of HCC represents the main reason for the increasing use of gadoxetic acid in cirrhotic patients[10-12]. The ability to detect HCC with gadoxetic acid depends on the differences in hepatocellular contrast uptake between HCC and the surrounding liver[4]. On hepatobiliary phase, HCCs are typically hypointense due to the absence of functioning hepatocytes, while the liver parenchyma enhances due to hepatocellular uptake of gadoxetic acid. Consequently, HCC to liver contrast and HCC detection rate are increased[4].

Hepatobiliary phase hypointensity also helps differentiate HCCs from dysplastic and regenerative nodules. Since hepatocellular uptake of gadoxetic acid decreases during hepatocarcinogenesis, hepatobiliary phase hypointensity suggests a diagnosis of HCC over that of dysplastic and regenerative nodules, which are typically iso- or hyperintense[13-16]. Typical imaging appearance of HCC includes moderate arterial enhancement and venous wash-out[17]. Using these criteria, however, several small HCCs can be missed because of absence of venous wash-out or, more rarely, arterial enhancement[18]. The hypointensity on hepatobiliary phase helps to correctly characterize small HCCs[13-16,19]. Hepatobiliary phase hypointensity, however, is not specific for the diagnosis of HCC because it can be found in any non-hepatocyte containing lesion (*e.g.*, hemangiomas, cholangiocarcinomas, metastases)[20].

Another application of gadoxetic acid is the pre-operative evaluation of patients scheduled for liver resection[21,22]. Recent studies have reported that quantitative analysis of hepatocellular uptake of gadoxetic acid can be used to estimate liver function and to predict the risk of liver failure after major hepatic

resection[21,22]. Hepatocellular uptake of gadoxetic acid correlates with indocyanine green clearance and uptake of radiopharmaceutical agents[22,23]. The advantages of gadoxetic acid over traditional methods, such as indocyanine green clearance and hepatic scintigraphy with radiopharmaceutical agents, include anatomic resolution (*i.e.*, liver function can be evaluated at segmental or subsegmental level) and the absence of ionizing radiation[24].

OPTIMAL STUDY PROTOCOL OF THE LIVER

An ideal MRI liver protocol should evaluate both liver parenchyma and vessels and should aid in detection and characterization of hepatic lesions. Typically, MRI liver protocol includes T2-weighted turbo or fast spin-echo (with and without fat saturation) sequences, gradient-recalled echo (GRE) T1-weighted in- and opposed-phase sequence, diffusion-weighted (DW) sequence, and contrast-enhanced three-dimensional T1-weighted GRE sequence with fat suppression. Field-strength magnets of 1.5 Tesla or greater are recommended to obtain high-quality liver imaging[25]. Contrast administration should be performed through a power injector. The use of a saline solution is strongly recommended because it reduces the dose of contrast material remaining in the dead space (*e.g.,* the brachial vein) and shortens the arrival time of contrast material in the hepatic arteries[10]. Contrast enhanced images are obtained on vascular, transitional, and hepatobiliary phases[26]. Vascular phases include the late hepatic arterial and portal venous phases[26]. Late hepatic arterial phase is crucial to detect and characterize hypervascular lesions[27]. Demonstration of moderate enhancement of intrahepatic portal veins, slight enhancement of liver parenchyma, and no enhancement of hepatic veins indicate an appropriate timing[28]. Achieving an adequate arterial phase with gadoxetic acid is more challenging than with conventional extra-cellular contrast materials. Due to the higher T1-relaxivity, gadoxetic acid has one-half lower contrast volume and one fourth lower Gd-content per kg than those of conventional extra-cellular contrast materials[29]. Thus, gadoxetic acid injection duration and time to peak aortic enhancement are shorter than those of conventional extra-cellular contrast materials[29]. In addition, the administration of gadoxetic acid has been associated with acute self-limited dyspnea, and consequent severe motion artifacts[30]. By definition, acute self-limited dyspnea is limited to the hepatic arterial phase images, and respiratory motion artifacts are absent in other sequences[30]. The exact cause remains unknown. A relationship between higher gadoxetic acid doses and chronic obstructive pulmonary disease has been reported[31]. Because the dyspnea is transient (10-20 s), a potential solution in order to overcome the artifacts is to acquire more than one arterial phase image. This approach is advantageous because: (1) acquisition of a greater number of phases increases the likelihood to obtain at least one diagnostic arterial phase image; and (2) reducing the acquisition time of each phase minimizes the opportunity for motion[30].

There are methods for achieving an optimal an optimal hepatic arterial phase. The most frequently used is a fixed delay (approximately 25-30 s) between the start of contrast injection and data acquisition. This method, however, is often inadequate because it does not take into account injection- or patient-related factors (*e.g.*, cardiac output) that influence circulation time. Indeed, arterial phase images are frequently obtained either too early (*i.e.*, before portal venous enhancement) or too late (*i.e.*, when contrast is already in the hepatic veins)[32]. Another option is the test bolus technique, in which a small test bolus (1-2 mL) of contrast material is injected to calculate contrast material arrival time. Although this technique is effective with extra-cellular contrast materials, it is not recommended in gadoxetic acid enhanced MRI because hepatocellular uptake of the bolus can increase liver signal intensity, and the removal of bolus volume from the pre-filled syringe can leave insufficient contrast to administer during the dynamic phases of the study. The use of a fluoroscopic system (MR SmartPrep, GE Medical Systems, Milwaukee, WI, United States; CARE Bolus, Siemens Medical Solutions, Erlangen, Germany; Bolus-Track, Philips Medical Systems, Best, The Netherlands) is preferable[10]. This technique is based on real-time monitoring of the bolus arrival at the level of the vessel of interest (typically the suprarenal abdominal aorta) with a 2D fluoroscopic sequence. Arterial phase acquisition can be started manually or automatically with a trigger threshold. The optimal scan delay for late hepatic arterial phase is 15-20 s after the peak aortic enhancement, which corresponds to the time necessary to synchronize the arrival of contrast material in the main portal vein with central k-space filling[26].

The injection of contrast material breaks k-space homogeneity and can cause truncation artifacts[33]. These artifacts appear as dark or bright lines at interfaces between high and low signal intensity structures (*e.g.*, enhanced arteries and surrounding liver parenchyma) and alter anatomic details of structures[34]. Several methods of minimizing truncation artifacts truncation artifacts have been proposed. One option is to use a larger volume of contrast material by diluting gadoxetic acid with saline[33]. Alternatively, a slow (1 mL/s) injection rate, which results in natural dilution of the contrast in the vascular space, can be used[35]. In addition, to increase k-space homogeneity, the larger contrast volume provides a wider temporal window of hepatic arterial phase. Tamada *et al*[36] compared arterial phase images obtained with three different techniques: diluted gadoxetic acid administered at conventional rate of 3 mL/s; undiluted gadoxetic acid administered at conventional rate of 3

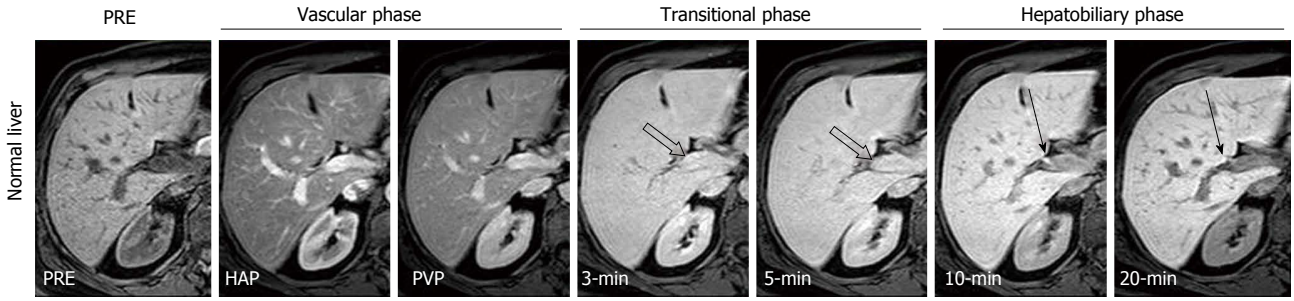

Figure 1 Gadoxetic acid contrast-enhanced magnetic resonance images obtained in a 46-year-old woman with normal liver. Contrast-enhanced magnetic resonance images show a stepwise intensity increase of the liver parenchyma from the hepatic arterial phase to hepatobiliary phase. On hepatic arterial and portal venous phases (vascular phase), the intrahepatic vessels show intense and homogeneous enhancement. On 3 min late and 5 min late phases (transitional phase), the intrahepatic vessels (open arrows) show isointensity to the liver, indicating the transition of gadoxetic acid from the extra-cellular spaces to the hepatocellular-spaces. On 10 min and 20 min phase (hepatobiliary phase), the intrahepatic vessels show hypointensity to the liver, while the bile ducts (arrows) show hyperintensity; these findings indicate an adequate hepatobiliary phase. Also note kidney hypointensity to the liver, which indicates normal hepatobiliary elimination of gadoxetic acid and adequate hepatobiliary phase. PRE: Precontrast; HAP: Late hepatic arterial phase; PVP: Portal venous phase.

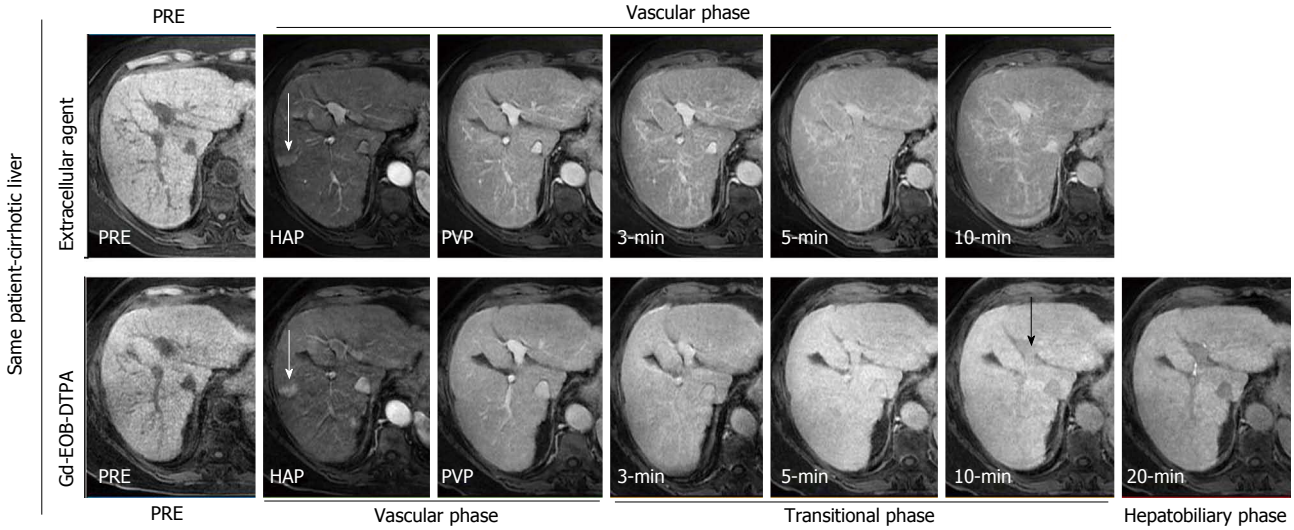

Figure 2 Intraindividual differences in hepatic enhancement in cirrhotic liver between extra-cellular contrast agent (top row) and gadoxetic acid (bottom row) in a 69-year-old woman with hepatitis C virus-related cirrhosis. On contrast-enhanced magnetic resonance (MR) images obtained with an extra-cellular agent liver enhancement peaks on portal venous phase and then slightly decreases. On contrast-enhanced MR images obtained with gadoxetic acid, liver enhancement shows a stepwise increase from the hepatic arterial phase to the 20 min phase. Vascular enhancement is more prolonged with extra-cellular agent than with gadoxetic acid, indicating a slower vascular elimination. On 10 min, the intrahepatic vessels (black arrow) show slight hypointensity to the liver, and the bile ducts are not opacified. These findings indicate hepatic dysfunction and a prolonged transitional phase. Also, note a wedge shaped enhancing area in the hepatic arterial phase (white arrow), with lack of washout on portal venous phase and isointensity on hepatobiliary phase, due to arterioportal shunt. PRE: Precontrast; HAP: Late hepatic arterial phase; PVP: Portal venous phase.

mL/s; and undiluted gadoxetic acid administered at a rate of 1 mL/s. They concluded that the injection rate of 1 mL/s with undiluted gadoxetic acid was preferable to the other two methods[37]. Portal venous phase is acquired 50-70 s after gadoxetic acid injection. On portal venous phase, the liver parenchyma shows intense enhancement, and the portal and hepatic veins are fully and maximally enhanced[38] The interval time (2-5 min after gadoxetic acid injection) between perfusion phase and hepatobiliary phase is termed "transitional phase", and, therefore, should not be confused with or referred to as the equilibrium phase that is typically obtained at the same time delay with extracellular contrast agents[37] (Figures 1 and 2). The transitional phase is obtained 3 min after the start of contrast injection[26]. Gadoxetic acid shows uptake by hepatocytes through a canalicular multispecific organic anion transporting polypeptide 1B3 (OATP1B3) as early as 90 s after contrast injection, but this process takes several minutes before all contrast is taken up by hepatocytes. Thus, gadoxetic acid "transitates" from interstitial space to intracellular space. That is why we refer to this phase as the transitional phase, indicating the transition of gadoxetic acid from the extra-cellular space to the hepatocellular space[37]. In contrast, extra-cellular contrast materials are equally distributed between vascular spaces and interstitial spaces. Hepatocellular uptake of gadoxetic acid explains higher signal intensity of liver parenchyma with gadoxetic acid than with extracellular contrast materials[39]. Earlier elimination of gadoxetic acid from the vessels leads to earlier de-enhancement and, therefore, lower signal

intensity of intrahepatic vessels with gadoxetic acid than with extra-cellular contrast materials (Figure 2)[39].

Hepatobiliary phase is acquired 10-20 min after the start of contrast injection. Since the injection of gadoxetic acid does not compromise tissue contrast on T2-weighted images and diffusion-weighted images, these sequences can be acquired in the interval between the 3 min phase and the hepatobiliary phase, thus reducing the total examination time[40-42]. DW images can help to differentiate hypovascular HCC from high-grade dysplastic nodules and can predict the progression of hypovascular hypointense nodules on hepatobiliary phase into hypervascular HCC[43,44]. That is, hyperintensity on high-b-value DW images suggests a diagnosis of HCC and is strongly associated with progression of hypovascular nodules into hypervascular HCC[43,44]. The adjunct of DW images, however, does not significantly improve the diagnostic accuracy of MRI with hepatobiliary contrast materials in the detection of HCC[45,46]. Most small HCCs are imperceptible on DW images because they have cellular density and microscopic architecture relatively similar to that of surrounding cirrhotic liver[46].

DIFFERENCES IN PHASES OF ENHANCEMENT BETWEEN GADOXETIC ACID AND EXTRA-CELLULAR CONTRAST MATERIALS

Although gadoxetic acid allows dynamic imaging during the hepatic arterial, portal venous, and 3 min phases, some enhancement characteristics are different from those of extracellular contrast materials[1,39] (Figure 2). Gadoxetic acid shows a biphasic enhancement pattern in the liver[1]. The first phase (arterial + portal venous) is due to distribution in the vascular compartment. The second phase is due to hepatocellular uptake of gadoxetic acid by the canalicular multispecific OATP1B3 and starts 90 s after injection[1]. Extra-cellular contrast materials distribute in the extracellular fluid compartments, and, as the name implies, they are not taken up by the hepatocytes[1]. Liver enhancement peaks on portal venous phase and then decreases[39]. Vascular enhancement is higher and longer with extracellular contrast materials than with gadoxetic acid[39]. It has been reported that, on hepatic arterial phase, aorta and liver parenchymal enhancement is weaker[39]. Since most HCCs are hypervascular, this can influence their detection and characterization[1,39]. On portal venous phase, the signal intensity of liver parenchyma is comparable between gadoxetic acid and extra-cellular contrast materials, but the signal intensity of portal vein is lower with gadoxetic acid than with extra-cellular contrast materials[39]. Thus, the evaluation of portal and hepatic veins can be suboptimal with gadoxetic acid[12]. Since HCC invasion into portal or hepatic vein and portal vein thrombosis influence treatment options and can preclude surgical resection and liver transplantation, vascular evaluation can reduce the advantages of gadoxetic acid.

DIFFERENCES IN PHASES OF ENHANCEMENT BETWEEN CIRRHOTIC AND NORMAL LIVER WITH GADOXETIC ACID

Cirrhosis is characterized pathologically by distortion of hepatic architecture due to marked bridging hepatic fibrosis and regenerative nodule formation[47]. The number of normal hepatocytes is reduced, and biliary excretion is impaired[34,48]. Cirrhosis alters liver perfusion with a reduction in portal inflow and a compensatory increase of arterial inflow[11]. Thus, on hepatic arterial phase, liver enhancement is higher in cirrhotic patients than in normal-liver patients[49]. On portal venous phase, however, liver enhancement is superimposable in cirrhotic patients and normal-liver patients[49]. At 3 min and in the hepatobiliary phases, liver enhancement is higher in normal patients than in cirrhotic patients and shows an inverse correlation with the severity of cirrhosis[49]. This is because hepatic fibrosis and the reduction in the number of functioning hepatocytes decrease the hepatocellular uptake of gadoxetic acid[49]. Pharmacokinetic analysis demonstrated that liver signal intensity shows a stepwise increase from the hepatic arterial phase to the hepatobiliary phase in patients with normal liver and in patients with Child-Pugh class A and B cirrhosis (Figure 1); on the other hand it does not significantly change from portal venous phase to 20 min hepatobiliary phase in patients with Child-Pugh class C cirrhosis[49] (Figure 3). The consequence is that oftentimes, at 20 min, the vessels will not be "dark" enough in patients with Child-Pugh class C cirrhosis, resulting in a suboptimal hepatobiliary phase. Thus, in our practice, acquisition of hepatobiliary phase beyond the conventional 20 min delay may be useful in patients with impaired hepatic function in order to allow the hepatocytes more time to take up contrast from the extracellular space[50,51]. Conversely, in normal-liver patients, a hepatobiliary delay of 10 min after gadoxetic acid injection is sufficient[52]. Unlike normal liver, cirrhotic liver can show heterogeneous enhancement on the hepatobiliary phase, which can further complicate the detection and characterization of hepatic nodules[49]. The heterogeneity directly correlates with Child-Pugh class[49]. Enhancement of biliary tree is delayed in patients with cirrhosis compared with normal-liver patients[48].

Tschirch *et al*[52] compared the visualization of biliary tree between cirrhotic patients and normal-liver patients and found that 16/40 (40%) cirrhotic patients showed sufficient visualization of the biliary tree within 30 min of injection, and 21/40 (53%) cirrhotic patients showed sufficient visualization of the biliary tree within 180 min of injection. In contrast, in their series, all

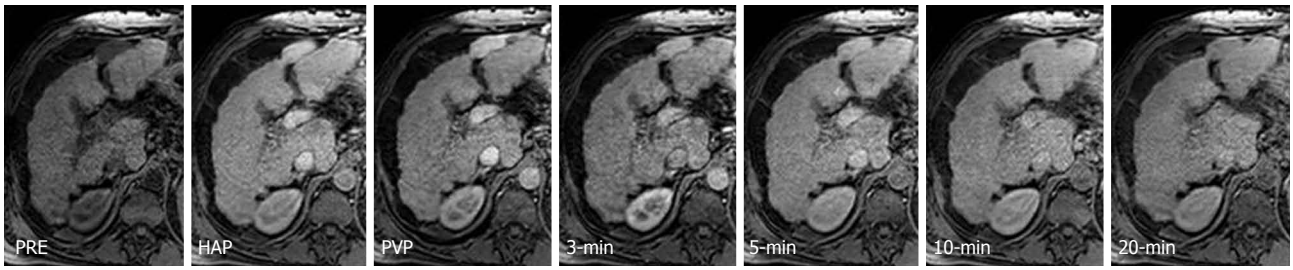

Figure 3 Gadoxetic acid contrast-enhanced magnetic resonance images obtained in a 57-year-old man with Child-Pugh C hepatitis C virus-related cirrhosis. Contrast-enhanced magnetic resonance images show slight decrease of liver enhancement after the portal venous phase. On hepatic arterial and portal venous phases, the intrahepatic vessels show intense and homogeneous enhancement, which persists on 3 min, 5 min, and 10 min phase. On 20 min phase, the intrahepatic vessels show isointensity to the liver. Prolonged retention of the contrast in intrahepatic vessels indicates impaired hepatic function and an inadequate hepatobiliary phase. Twenty minutes phase corresponds in this case to the transitional phase observed in normal liver patient due to prolonged retention of gadoxetic acid in intrahepatic vessels. Also, note that the kidney shows isointensity to the liver on 10 min and 20 min phases, indicating a compensatory increase of renal elimination of gadoxetic acid and an inadequate hepatobiliary phase. PRE: Precontrast; HAP: Late hepatic arterial phase; PVP: Portal venous phase.

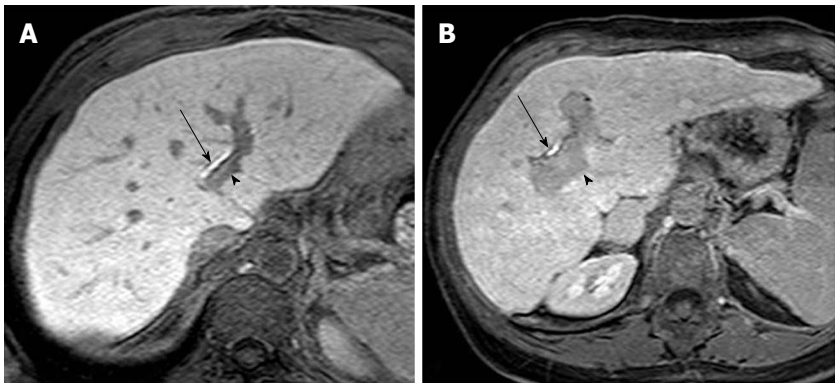

Figure 4 Twenty-minute hepatobiliary phase gadoxetic acid enhanced magnetic resonance imaging obtained in a 67-year-old man with Child-Pugh class A hepatitis C virus-related cirrhosis (A) and in a 67-year-old woman with Child-Pugh class B hepatitis C virus-related cirrhosis (B). A: The liver shows high signal intensity compared with the portal vein (arrowhead), which shows hypointensity; B: The liver shows relative high signal intensity compared with the portal vein (arrowhead), which shows "less" hypointensity if compared with A. Visual comparison of signal intensity of the liver relative to the portal vein can be used to evaluate adequacy of hepatobiliary phase. Enhancement of bile ducts, noted in both A and B (arrows), cannot be used alone to indicate adequacy of hepatobiliary phase.

normal-liver patients showed sufficient visualization of the biliary tree within 30 min of injection[48].

ADEQUACY OF HEPATOBILIARY PHASE

In patients with normal hepatic function, gadoxetic acid is equally eliminated by biliary excretion and glomerular filtration[6]. Impaired hepatic function results in a compensatory increase of renal elimination and more prolonged plasma half-life of gadoxetic acid in cirrhotic patients than in normal-liver patients[36]. The consequence is typically a decrease of contrast between liver parenchyma and portal vein[53]. Visual evaluation of the signal intensity of the liver relative to the portal vein or kidney can help radiologists assess adequacy of the hepatobiliary phase[34,38]. Specifically, brighter signal intensity of the liver parenchyma compared with the portal vein and kidney indicates an adequate hepatobiliary phase, while persistent contrast within the portal vein and brighter or equal signal intensity of the kidney compared with the liver parenchyma indicates an inadequate hepatobiliary phase[36,39] (Figures 3 and 4). Opacification of the biliary tree shows no correlation with the severity of cirrhosis and cannot be used alone to evaluate adequacy of the hepatobiliary phase[48] (Figure 4).

The uptake of gadoxetic acid does not depend only on the hepatic function but also on the hepatic blood flow[33]. Motosugi *et al*[33] reported that most patients with Child Pugh Class A cirrhosis and inadequate hepatobiliary phase had considerable arterial-portal and portal-systemic shunts. The shunts decrease the hepatic blood flow and hepatic retention of gadoxetic acid[33]. Other causes of reduced hepatobiliary phase enhancement include severe steatosis (Figure 5), hepatic fibrosis, and iron overload[54-57]. An inadequate hepatobiliary phase may impair detection and characterization of focal liver lesions because the contrast between focal liver lesions and the liver parenchyma is reduced[58]. These patients should be evaluated with alternative modalities, such as contrast-enhanced CT and contrast-enhanced ultrasound, in order to avoid misdiagnosis. To date, however, no liver function test can predict whether the hepatobiliary phase result will be adequate.

Recent studies have demonstrated that increasing the flip-angle from 10°-15° to 30°-40° can improve detection and conspicuity of focal hepatic lesion,

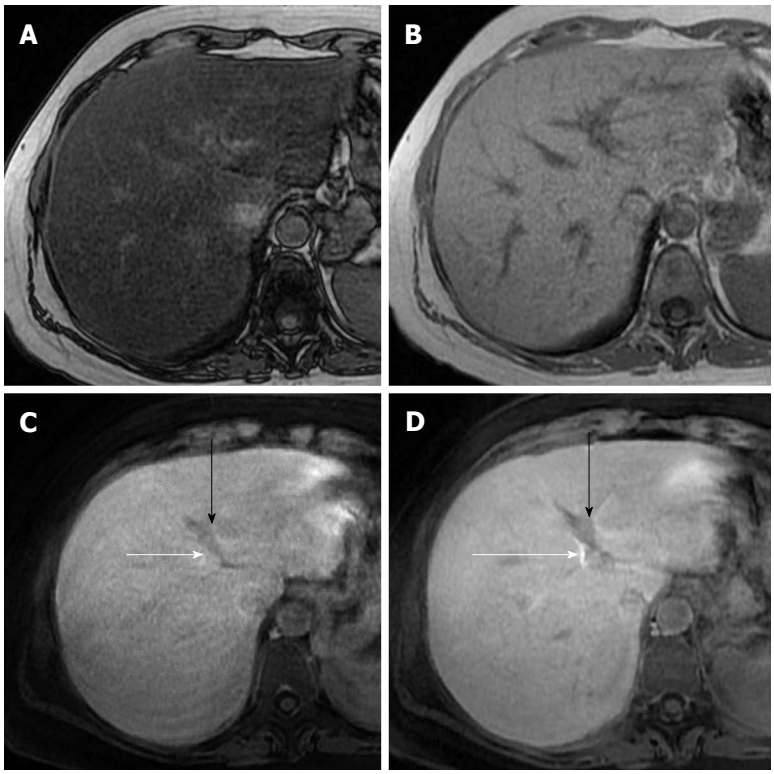

Figure 5 Reduced hepatobiliary phase enhancement due to severe hepatic steatosis in a 42-year-old woman with hepatitis C virus-related chronic hepatitis. A, B: T1-weighted gradient-echo images show diffuse signal intensity decrease of the liver on out-of-phase (A) image compared with that on the in-phase image (B), indicating severe hepatic steatosis; C: On 10 min hepatobiliary phase, gadoxetic acid enhanced magnetic resonance imaging, left portal vein (black arrow) shows iso- to hypointensity to liver parenchyma; D: On 20 min hepatobiliary phase left portal vein shows slight hypointensity to liver parenchyma. Enhancement of bile ducts (white arrows) is less intense on 10 min hepatobiliary phase than that on 20 min hepatobiliary phase, indicating delayed biliary elimination of gadoxetic acid.

particularly of small lesions[44-46]. Larger flip angle maximizes T1-contrast and results in better differentiation between tissues with short T1-relaxation times, such as liver parenchyma with gadoxetic acid uptake and tissues with long T1-relaxation times, such as lesions without functioning hepatocytes[59-61]. Larger flip angle, however, increases specific absorption rate (SAR) in patient tissue[59].

CONCLUSION

Gadoxetic acid enhanced liver MRI is emerging as a powerful tool in the diagnostic workup of cirrhotic patients and provides unique information related to lesion vascularity and hepatobiliary function. Use of gadoxetic acid improves detection and characterization of focal liver lesions, and hepatocellular uptake can be used as a measure of liver function. Thus, radiologists involved in liver imaging need to be familiar with the state-of-art MRI study protocol of the liver and the unique characteristics of gadoxetic acid.

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TOPIC HIGHLIGHT

2016 Cirrhosis: Global view

Left ventricular function assessment in cirrhosis: Current methods and future directions

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Abstract

Cirrhotic cardiomyopathy has been defined as a chronic cardiac dysfunction in patients with cirrhosis characterized by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease. Non-invasive cardiovascular imaging modalities play a major role in unmasking systolic and diastolic dysfunction in patients with cirrhosis. Echocardiography has been the most commonly used modality for assessing myocardial function in these patients. Conventional echocardiographic indices rely on several assumptions that may limit their applicability in patients with a hyperdynamic circulation. Newer imaging modalities may contribute to a more accurate diagnosis of cardiovascular abnormalities in cirrhotic patients, thereby influencing clinical management. We aimed to review the different non-invasive imaging technologies currently used for assessing left ventricular systolic and diastolic function in cirrhosis, as well as to describe new imaging modalities with potential clinical applicability in the near future.

Key words: Cirrhosis; Cardiomyopathy; Echocardiography; Magnetic resonance imaging; Systolic function; Diastolic function; Deformation imaging

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Core tip: Cardiac dysfunction has been documented in cirrhosis. Conventional non-invasive methods are frequently used to detect abnormalities. Newer imaging techniques have been developed and can contribute to a more accurate diagnosis of cirrhotic cardiomyopathy. However, it is essential to understand the strengths and limitations of every modality in order to correctly interpret the results. Currently applied methods for assessing left ventricular myocardial function as well as future perspectives are reviewed.

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INTRODUCTION

Cardiovascular dysfunction in patients with cirrhosis has been recognized for more than sixty years[1,2]. These abnormalities were initially attributed exclusively to the effects of alcohol; however findings from animal and clinical studies performed during the last decades, lent support to the existence of a specific cardiomyopathy in cirrhosis, irrespective of its etiology[3]. Based on these reports, in 2005, cirrhotic cardiomyopathy was defined as a "chronic cardiac dysfunction in patients with cirrhosis characterized by impaired contractile responsiveness to stress and/or altered diastolic relaxation with electrophysiological abnormalities in the absence of other known cardiac disease". Several criteria for diagnosing this entity were proposed, relying mostly on non-invasive assessment of myocardial function[4-6].

In the last years, the role of cardiac imaging in the management of cardiovascular diseases has been increasingly important. The development of newer imaging modalities resulted in an improvement in diagnostic accuracy and prognostic information thereby influencing clinical management of several cardiac disorders. Although the role of these methods in the diagnostic work-up of cirrhotic cardiomyopathy is still uncertain, several recent studies suggested their utility in unmasking myocardial dysfunction in this population[7,8]. The aim of this paper is to review the different non-invasive imaging technologies currently used for assessing left ventricular systolic and diastolic function in cirrhosis, as well as to describe new imaging modalities with potential clinical applicability in the near future.

SYSTOLIC FUNCTION

Ejection fraction (EF) is the most widely used parameter of global left ventricular systolic function. It is calculated from end-systolic and end-diastolic volumes, which can be estimated by different methods.

Echocardiography was the most commonly used modality for assessing EF in studies in cirrhosis[9-11]. The disk summation method in two orthogonal planes (modified Simpson's rule; Figure 1) is still the method of choice for calculating ejection fraction in recent recommendations[12]. Volumes and EF derived from linear dimensions may be very inaccurate in several conditions and should not be used. Three-dimensional echocardiography (3DE) is becoming more available and increasingly used in clinical practice (Figure 1). Left ventricular volumes derived from 3DE do not rely on geometrical assumptions and may be more accurate and reproducible, when compared to cardiac magnetic resonance (CMR)[13-16]. Fully automated software is commercially available allowing fast online measurements and better reproducibility. However, 3DE is highly dependent on image quality and has lower temporal resolution than two-dimensional echocardiography (2DE); some ultrasound systems still require electrocardiogram gating and breath hold making it more prone to artifacts. These issues may limit its applicability in cirrhotic patients with tachycardia and/or unable to hold their breath[17]. Volumes obtained with 3DE are larger than 2DE-derived volumes and should not be used inter-changeably in serial measurements[12]. To the best of our knowledge, there are no studies comparing 3DE with 2DE or CMR in cirrhosis; hence, its validity in this specific setting remains unproven.

Cardiac magnetic resonance has evolved into the reference standard methodology for assessment of cardiac morphology and volumes[18,19]. Such as echocardiography it provides morphological and functional information, does not use ionizing radiation and has a better spatial resolution than 2DE. Volumes obtained with CMR are also larger than echocardiography-derived values[16,20-22]. The widespread use of this method is limited by availability and cost. Its use in cirrhotic patients may also be hampered by the need of repeated end-expiratory apneas for image acquisition and tachycardia (decreasing temporal resolution) or irregular heart rates. Current development of improved free breathing and short breath-hold sequences may soften some of these problems in the near future[23,24].

Computed tomography and single photon emission computed tomography (SPECT) are alternative modalities for calculating EF and some studies in cirrhosis have used SPECT to quantify LV function[25,26]. However, these methods are limited by low temporal resolution and radiation.

According to the current consensus, an EF of less than 52% in men and 54% in women, using 2DE, suggests systolic dysfunction. Reference values for 3DE may be different since there is less published data on normal subjects[12]. Higher cut-off values may also apply to CMR[27]. In the 2005 World Congress of Gastroenterology, a resting EF < 55% was proposed as a diagnostic criteria of systolic dysfunction. However, since EF is highly dependent on loading conditions, a higher cut-off value may need to be considered in patients with cirrhosis due to the peripheral vasodilatation and decreased afterload. This probably explains the finding of normal resting ejection fraction in the majority of the studies in cirrhosis[5,28-30].

NEWER INDICES OF SYSTOLIC FUNCTION

Although widely used in systolic function assessment, EF has several limitations. Ejection fraction is not

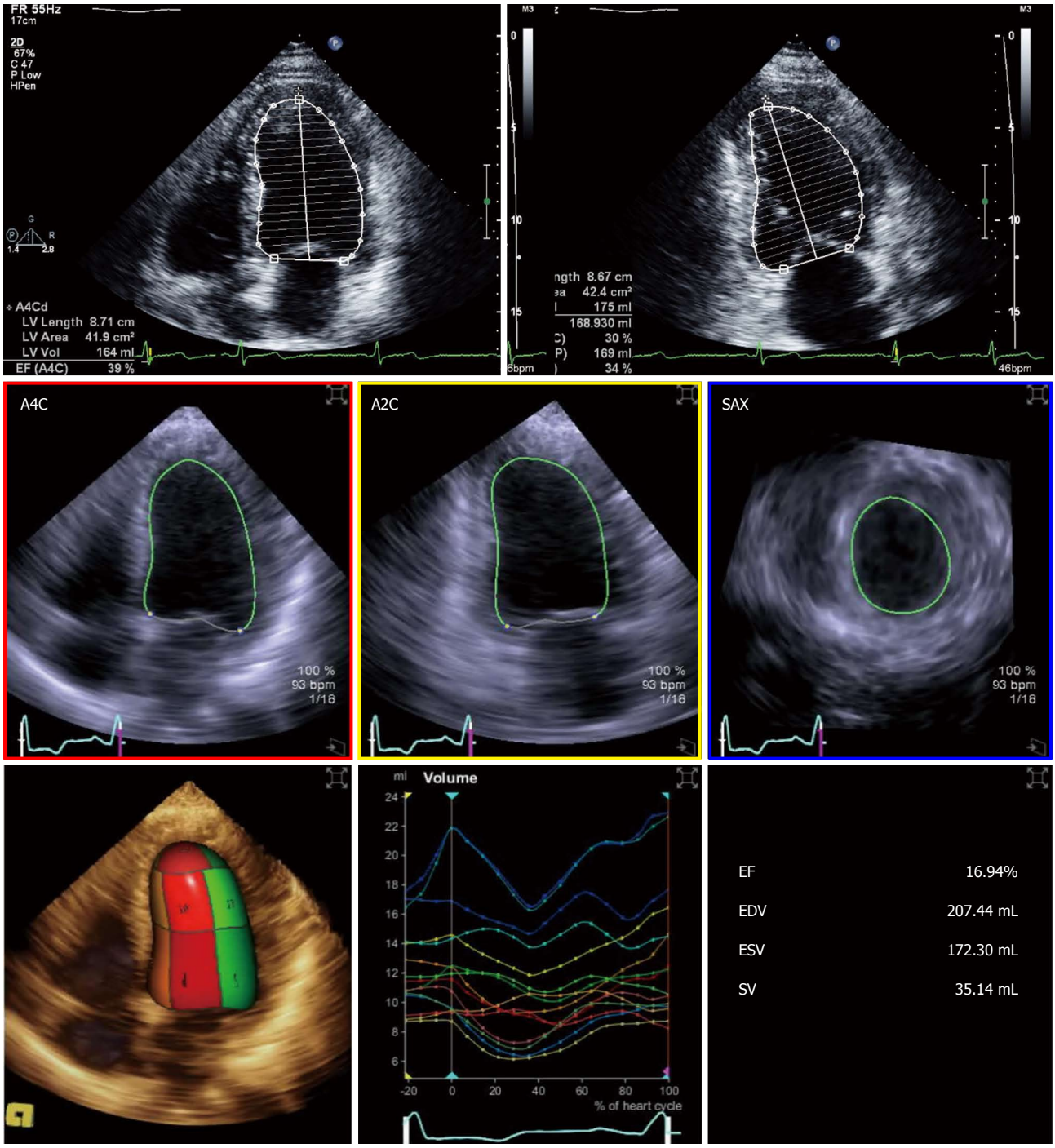

Figure 1 Ejection fraction determination using two-dimensional (biplane Simpson's method - upper panel) and three-dimensional echocardiography (fully automated software - lower panel).

an index of contractility and depends on loading conditions, heart rate and valvular function[31]. Besides, EF relies on accurate tracing of endocardial borders and the inter-observer agreement in different measurements can be modest[32].

Tissue Doppler imaging (TDI) and speckle tracking echocardiography are newer imaging modalities able to objectively quantify regional and global LV function. Deformation is computed from the spatial gradients of myocardial velocities (using TDI) or from the relative position of "speckles" within a myocardial region, along the cardiac cycle (speckle tracking). Strain (ε) is the fractional change in length of a myocardial segment relative to its original dimension, and is expressed as a percentage (%). Strain rate (SR) is the change in strain over a period of time and is usually expressed as 1/s or s^{-1}. Both TDI and speckle tracking have strengths and weaknesses, that are described in detail elsewhere[33,34]. Briefly, TDI is mainly limited by angle-dependency (making it unsuitable to assess circumferential motion) and by artifacts, while speckle tracking has lower temporal resolution, which may

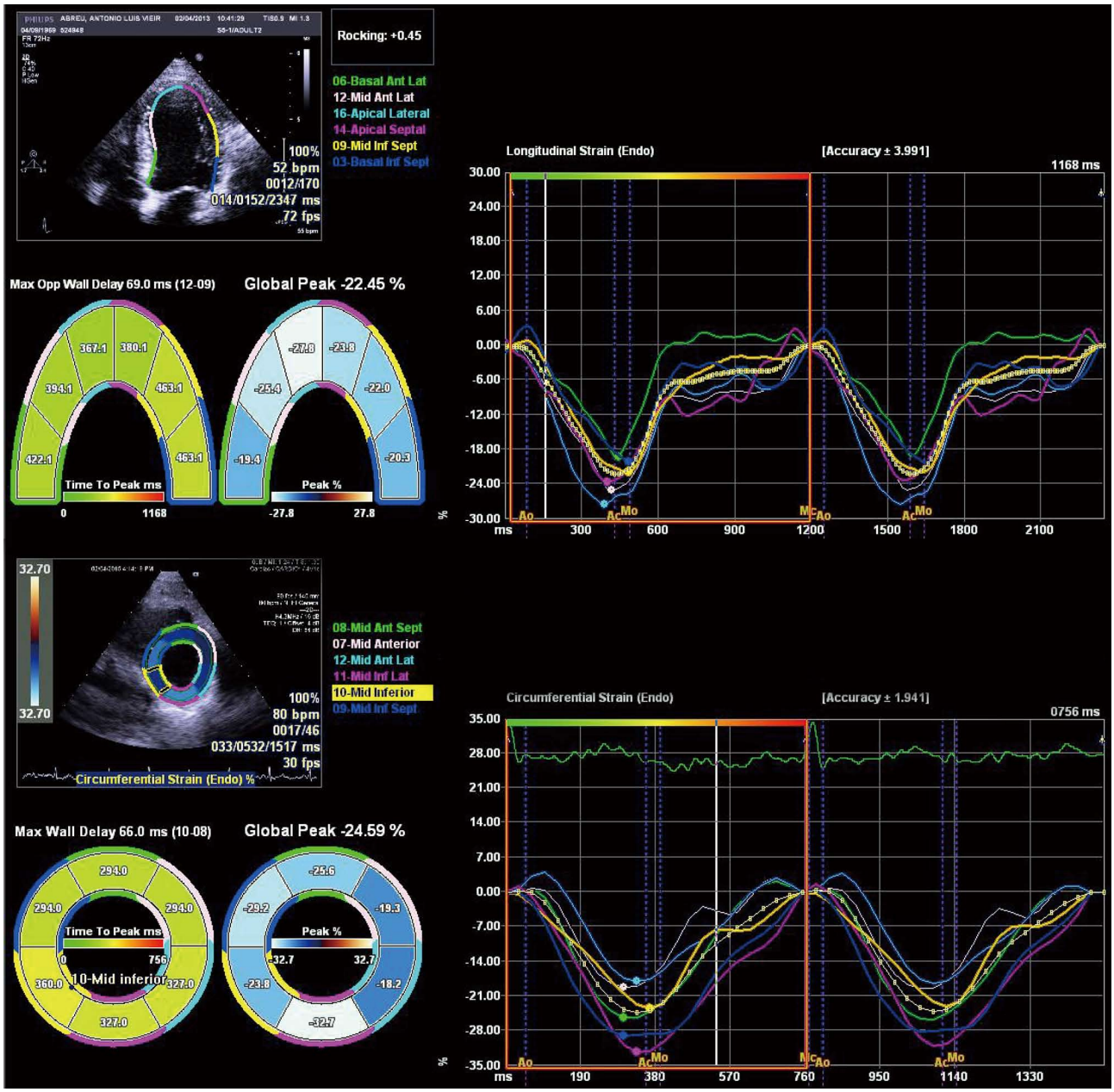

Figure 2 Left ventricular deformation analysis using speckle tracking echocardiography. Longitudinal (top panel) and circumferential (lower panel) strain curves are displayed.

limit its use in patients with higher heart rates (such as patients with decompensated cirrhosis) or in the assessment of short-lived events. Speckle tracking has the advantage of being able to quantify all the components of myocardial mechanics (longitudinal, circumferential and radial motion/deformation as well as rotation and torsion; Figure 2) within the image plane. It may also be more reproducible than TDI[35-39]. There is a large body of evidence supporting the clinical utility of myocardial deformation analysis in cardiovascular disease. These methods were successfully used in identifying subclinical myocardial dysfunction in different settings, in the improvement of the performance of stress echocardiography for diagnosing coronary artery disease, in the assessment of therapeutic interventions in cardiomyopathies and in the prediction of outcomes[40-48]. Left ventricular longitudinal dysfunction has also been previously documented in patients with cirrhosis, at rest[7,8]. Longitudinally oriented subendocardial fibers are more susceptible to damage than the radial fibers of the middle myocardium layer and this probably accounts for these findings. Loading conditions also influence strain and strain rate[49-51] and this should be taken into account when interpreting strain results in patients with cirrhosis, since the decreased afterload secondary to peripheral vasodilatation improves strain values. This may explain previous findings of strain values within the normal range in patients with decompensated cirrhosis as well as similar strain between hospitalized and ambulatory patients[7,8,52].

The presence of an abnormal response to exercise

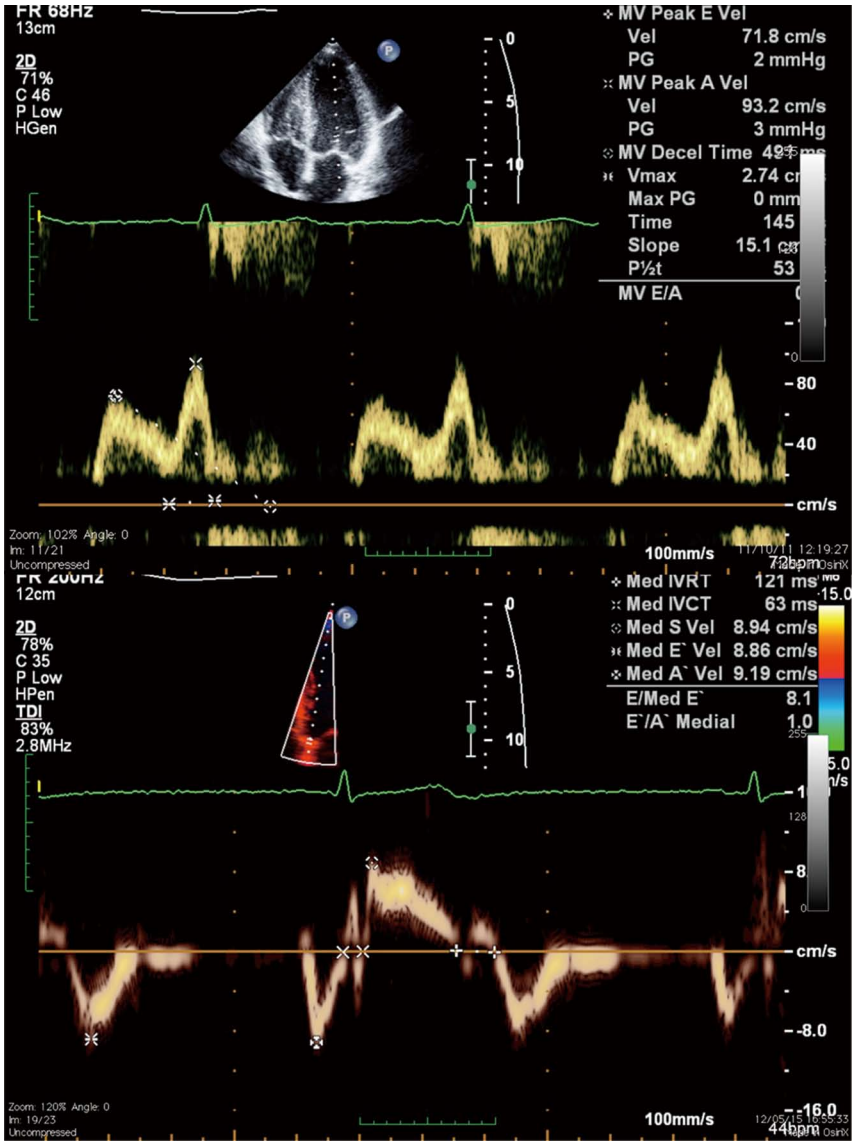

Figure 3 Mitral inflow velocities using pulsed-wave Doppler showing a impaired relaxation pattern (top), and tissue-Doppler derived mitral annulus velocities at the septal wall (bottom).

or pharmacological stress has been reported as a feature of cirrhotic cardiomyopathy[26,53-58]. Limitations of classical non-invasive parameters of systolic function also apply under stress (particularly pharmacological stress). Deformation may also be assessed during stress and abnormalities in the response of myocardial deformation to exercise have been found in heart failure patients, using speckle tracking[59]. The improvement in longitudinal strain under low-dose dobutamine may be lower in patients with cirrhosis when compared to a control group suggesting that longitudinal dysfunction can contribute to the inotropic incompetence previously documented in these patients[60].

A major limitation to the widespread use of speckle tracking in routine clinical practice is the significant variability that exists among vendors and software packages that prevent the definition of normal reference values. Likewise, abnormal strain variations in follow-up echocardiographic studies cannot be safely determined when using different analysis software[61,62]. An effort to implement standardization in strain imaging is currently underway and will hopefully reduce intervenor variability of strain[63].

DIASTOLIC FUNCTION

Diastolic dysfunction (DD) has been reported as a common finding in patients with cirrhosis. Abnormalities in membrane receptor function and intracellular signaling pathways, as well as changes in contractile proteins and extracellular matrix composition are probably involved in the pathogenesis of DD in cirrhosis[64-68].

Non-invasive assessment of DD has classically relied on the echocardiographic analysis of mitral inflow pattern using pulsed-wave Doppler (Figure 3). In the presence of mild DD, early diastolic filling is decreased

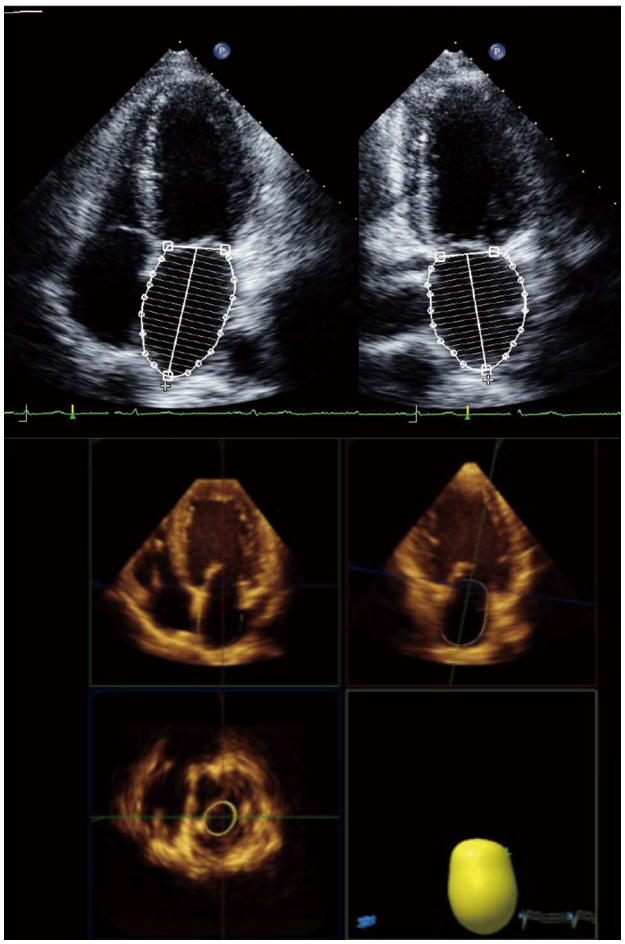

Figure 4 Left atrial volume quantification using two-dimensional (biplane Simpson's method - upper panel) and three-dimensional echocardiography (lower panel).

as a result of delayed LV relaxation and atrial contraction becomes a more important contributor to left ventricular filling. This impaired relaxation pattern (grade Ⅰ DD) is characterized by a decrease in E wave velocity, prolongation of E-wave deceleration time, and an increase in A wave velocity resulting in an inverted E/A ratio (< 1). With worsening DD, the increase in left atrial pressure restores the early diastolic pressure gradient, increasing E wave velocity; on the other hand, LV pressure at the end of diastole is higher, so that the contribution of atrial contraction (A wave) is reduced. So, in grade Ⅱ DD, mitral flow is similar to that in the normal individual (hence called pseudonormal pattern), with the E wave again greater than the A wave. The Valsalva maneuver, which decreases preload, can be used to differentiate a normal from a pseudonormal pattern since the latter is changed to an impaired relaxation pattern during the maneuver. With even more severe DD (grade Ⅲ), there is a marked elevation in LA and LV pressures and most filling occurs during early diastole. In this restrictive pattern, E wave velocity is increased, E wave deceleration time is very short (< 160 mseg) and the E/A ratio is > 1.5.

Diastolic dysfunction, as defined in the 2005 World Congress of Gastroenterology (E/A ratio < 1.0, deceleration time > 200 ms and isovolumetric relaxation time > 80 ms, is highly prevalent in patients with cirrhosis[10,69,70]. An association between liver disease severity and DD and an improvement in DD after paracentesis has also been reported. Earlier studies also suggested that DD was related to the liver disease severity and improved after paracentesis[10,71]. More importantly, an association between an E/A ratio < 1 and increased mortality and slower mobilization of ascites after transjugular intrahepatic portosystemic shunt (TIPS) insertion has been suggested[72,73].

However, mitral inflow-based assessment of diastolic function has several limitations that should be taken into account[74-78]. Different loading and heart rate can significantly change the E/A ratio and DT, even in normal subjects[79-81]. This can be a major issue in cirrhotic patients, due to the blood pooling in the splanchnic bed and reduced preload which, along with faster heart rates, may result in lower E/A ratios, regardless of the presence of impaired relaxation. This can also contribute to the association of this pattern with mortality since there is usually a direct relation between hyperdynamic circulation and disease severity[82]. Besides, impaired relaxation has a better prognosis than more advanced stages of DD, both in the general population and in heart failure patients[83,84].

Acknowledging the pitfalls of mitral-flow variables, recommendations for evaluating left ventricular diastolic function by echocardiography have been issued in 2009[80]. According to this consensus document, tissue-Doppler diastolic velocities of the mitral annulus play a major role on DD assessment (Figure 3). Early diastolic annular velocity (E') is a sensitive parameter of myocardial relaxation[85-87]. E' is also a surrogate marker of the volume that enters the left ventricle during early diastole. Since E wave velocity reflects the pressure gradient between the left atrium and left ventricle, the E/E' ratio represents the volume that enters the ventricle for a given LA-LV pressure gradient. A high E/E' ratio means that there is a small volume change between the two chambers despite a high pressure gradient, reflecting diastolic dysfunction with an increase in left ventricular filling pressure[88,89].

Using a tissue-Doppler based approach, some studies have found a lower prevalence of DD in cirrhotic patients; furthermore, the prevalence of DD did not differ between disease stages[8,52,90,91]. The association between DD and prognosis also remains controversial, with conflicting results reported in different studies[52,92-94].

Although a clear advance in the diagnostic workup of DD, tissue-Doppler based parameters are not flawless, and E/E' may not adequately reflect left ventricular filling pressures in different settings. Hence, the diagnosis of DD should not rely on a

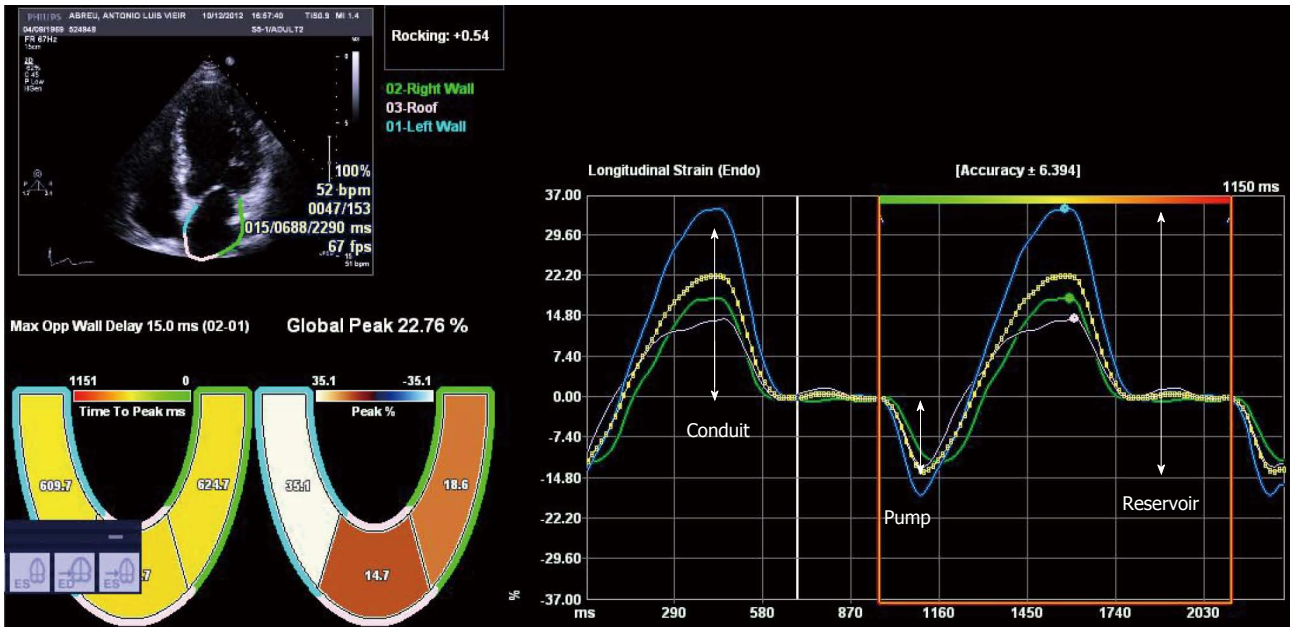

Figure 5 Left atrial deformation analysis using speckle tracking echocardiography. Reservoir, conduit and pump function of the left atrium during the cardiac cycle can be quantified from the strain curves.

single measurement and a multi-parameter approach (including tissue-Doppler mitral annulus velocities, pulsed-waved Doppler mitral inflow and pulmonary veins velocities, and left atrial volume) is usually recommended[80,95]. Unfortunately, the use of these more complex algorithms results in some variability in the classification of DD, even among expert echocardiographers[96,97]. This probably explains some of the differences in the prevalence of DD among more recent studies in cirrhosis.

Speckle tracking echocardiography can also provide information regarding diastolic function, through the analysis of strain rate during isovolumic relaxation, early filling and late diastole[33,85]. However, the lower temporal resolution of speckle tracking can limit the analysis of fast events (such as those during diastole), and there is no evidence to support its superiority over the already established TDI parameters[98].

Left atrial volume, preferably indexed to body surface area, is a mandatory measurement for the assessment of diastolic function. Left atrial volume index (LAVI) is strongly associated with the severity and duration of DD reflecting the cumulative effects of elevated filling pressures over time[83]. As for LV volumes and EF, the disk summation algorithm is the recommended method for calculating LAVI (Figure 4)[12]. An increase in LAVI has also been reported in cirrhosis, which has been interpreted as a marker of DD in these patients[29]. However, dilated atria may also be seen in patients with volume overload, anemia or other high-output states such as cirrhosis. We have previously found that, in a cohort of patients with cirrhosis of several etiologies, LAVI was associated with stroke volume, LV end-diastolic volume and hemoglobin and not with diastolic dysfunction, suggesting that atrial enlargement in cirrhosis may be related to loading conditions and should not be used as a marker of DD[99]. Speckle tracking echocardiography can also be used in the assessment of left atrial phasic volumes and deformation, providing information about left atrial function (Figure 5)[100,101]. Atrial dysfunction may be involved in the pathophysiology of heart failure with preserved ejection fraction and may be associated with symptom onset[102-105]. Left atrial longitudinal strain correlates better with LV filling pressures than LA volume or other echocardiographic indices such as the E/E' ratio[106,107]. A decrease in LA longitudinal strain has also been reported in patients with cirrhosis, and this seems to relate to abnormal relaxation in these patients[99].

Magnetic resonance can be used to study diastolic function[108]. Both mitral inflow and pulmonary vein velocities can easily be determined and a good correlation with echocardiography-derived indices has been demonstrated[109]. However, like echocardiographic variables, mitral inflow velocities calculated by magnetic resonance are heavily dependent on loading conditions and heart rate. Availability issues, costs, and lower temporal resolution (when compared to echocardiography) also hamper cardiac magnetic resonance limiting its use to patients in which echocardiography is non-diagnostic.

Magnetic resonance can also be used to detect myocardial fibrosis through the quantification of late gadolinium enhancement (LGE). A diffuse pattern of intramyocardial LGE has been previously described in patients with cirrhosis; the authors did not analyze a possible relation between LGE extent and DD indices[110]. The use of gadolinium-based contrast agents should be very cautious in patients with renal

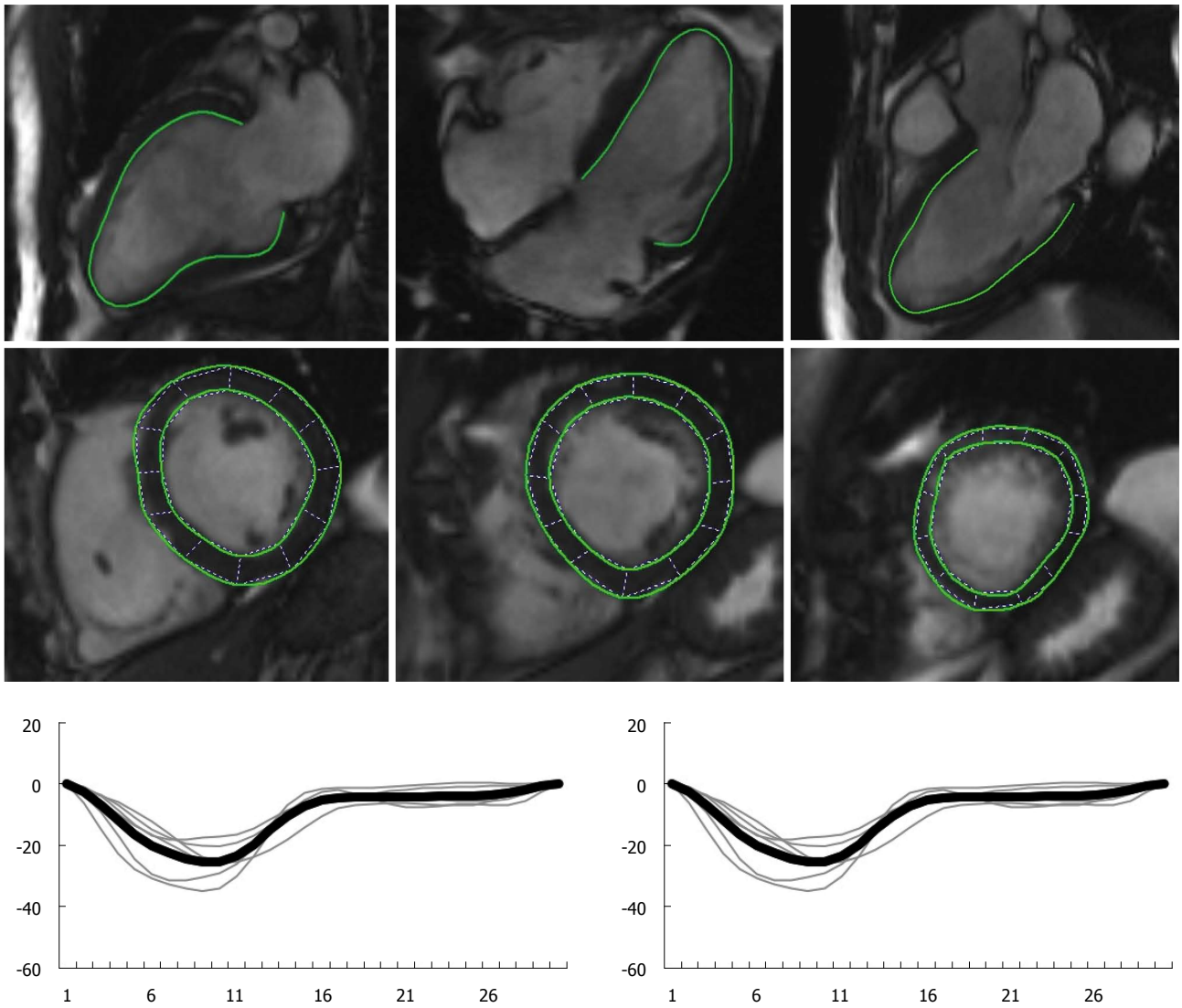

Figure 6 Left ventricular deformation analysis using magnetic resonance feature tracking.

failure due to case-reports of nephrogenic systemic fibrosis induced by older linear gadolinium chelates[111]. This may be an issue in patients with advanced cirrhosis and hepatorenal syndrome.

FUTURE PERSPECTIVES

Three-dimensional echocardiography will improve to allow the acquisition of single-heartbeat full-volume data sets with higher temporal and spatial resolution. Chamber volume quantification will become quicker, more simple and reproducible with the use of fully automated software (Figures 1 and 4). Three-dimensional speckle tracking can analyze the deformation of the heart from a single data set. Although limited by a lower temporal resolution, 3D speckle tracking use will result in a more complete and more accurate analysis of myocardial function[112].

Myocardial deformation can also be quantified with CMR. Systolic and diastolic strain rate, atrial deformation parameters and twisting/untwisting are indices of myocardial function that can be assessed with CMR[113-115]. Newer technologies, like feature tracking (a technique analogous to speckle tracking, which tracks tissue voxel motion of CMR cine images) will result in faster scans and post-processing analysis times (Figure 6).

T1 mapping is a new CMR application that quantifies T1 relaxation times for each myocardial pixel. With the use of gadolinium-based contrast agent, the extracellular volume fraction of the myocardium - a surrogate marker of the size of extracellular matrix - can also be quantified. This may allow for the detection and quantification of diffuse myocardial involvement in different disease processes. A detailed description of technical aspects of these new modalities can be found elsewhere[116,117]. T1 mapping has been a field of intensive research in the last few years. Its usefulness in diagnosing infiltrative diseases (such has amyloidosis, Fabry disease or iron overload) as well as the diagnostic and prognostic value of fibrosis quantification in heart failure, cardiomyopathies,

myocarditis or cardiac involvement in systemic diseases has been suggested in several studies[118-132]. Although never previously reported, its use in cirrhosis, conceptually, appears to be very promising. However, issues regarding standardization still preclude clinical use of T1 mapping and this technique is mainly in the research field[116].

In conclusion, these newer CMR technologies, along with classical volume determination, flow analysis and late enhancement quantification will allow a comprehensive evaluation of myocardial function in a single exam, not dependent on a good "acoustic window" for image acquisition and with no radiation.

CONCLUSION

A large number of parameters derived from different imaging modalities are currently available for the assessment of left ventricular function. Newer technologies will become widely available in the near future, allowing a detailed evaluation of myocardial function, and improving the diagnostic accuracy of the tests. However one must be aware of the limitations of every parameter in order to correctly interpret the results. On the other hand, the use of these new methods in cirrhosis has been limited to a few studies and further work is needed to evaluate their diagnostic performance and, more importantly, the impact on clinical management of this specific group of patients.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Genetic variation of hepatitis B virus and its significance for pathogenesis

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Abstract

Hepatitis B virus (HBV) has a worldwide distribution and is endemic in many populations. Due to its unique life cycle which requires an error-prone reverse transcriptase for replication, it constantly evolves, resulting in tremendous genetic variation in the form of genotypes, sub-genotypes, and mutations. In recent years, there has been considerable research on the relationship between HBV genetic variation and HBV-related pathogenesis, which has profound implications in the natural history of HBV infection, viral detection, immune prevention, drug treatment and prognosis. In this review, we attempted to provide a brief account of the influence of HBV genotype on the pathogenesis of HBV infection and summarize our current knowledge on the effects of HBV mutations in different regions on HBV-associated pathogenesis, with an emphasis on mutations in the preS/S proteins in immune evasion, occult HBV infection and hepatocellular carcinoma (HCC), mutations in polymerase in relation to drug resistance, mutations in HBV core and e antigen in immune evasion, chronicalization of infection and hepatitis B-related acute-on-chronic liver failure, and finally mutations in HBV x proteins in HCC.

Key words: Hepatitis B virus; Genotype; Variation; Pathogenesis

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Core tip: Due to the unique life cycle of hepatitis B virus (HBV) which requires an error-prone reverse

transcriptase for replication, it constantly evolves resulting in significant genetic variation in the form of genotype, sub-genotype, and mutations. A large number of publications on the relationship between HBV genetic variation and HBV-related pathogenesis have appeared in recent years. However, the progress in this field has not been reviewed. We have attempted to provide a brief account of the influence of HBV genotype and mutations in the different viral genome regions on HBV-associated pathogenesis. This review provides an overview for scientists working on HBV and related fields.

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INTRODUCTION

Hepatitis B virus (HBV) is the prototype member of a family of viruses called hepadnaviruses. It has a relaxed partially double-stranded circular DNA genome of approximately 3200 bases with four overlapping open reading frames (ORFs): pre-S/S (surface proteins), pre-C/C (pre-core/core), X (transcriptional co-activator) and P (DNA polymerase). The pre-S/S ORF is contained completely within the P ORF, but is translated in a different reading frame. ORFs C and X overlap the ORF P by 1/4 and 1/3 of their respective sequence lengths[1]. The preS/S ORF encodes three different, structurally related envelope proteins, which are synthesized from alternative initiation codons and termed Large (L), Middle (M) and Small (S) protein, respectively. The S protein [hepatitis B surface antigen (HBsAg)] consists of 226 amino acids (aa), and the M protein has an extra N-terminal extension of 55 aa, whereas the L protein has a further N-terminal extension of 108 or 119 aa depending on the genotype. The preC/C ORF codes for two distinct products derived from in-frame alternative initiation sites on the same transcript: the core protein forming the protein shell of the nucleocapsid [hepatitis B core antigen (HBcAg)] and the precore protein which is targeted into the cell's secretary pathway, processed at both ends and eventually found in the serum of infected individuals [hepatitis B e antigen (HBeAg)]. The X ORF encodes the small regulatory X protein, which is essential for viral replication, and plays roles in modulating host and viral gene expression. The P ORF encodes protein P, the viral DNA polymerase[2].

HBV relies on protein P, which is also a specialized reverse transcriptase (RT), to replicate its genomic DNA *via* a RNA intermediate[3]. Protein P consists of four domains: a terminal protein that is covalently linked to the DNA primer during negative-strand DNA synthesis, a spacer domain that is tolerant to mutations, the RT domain, and the ribonuclease H (RNase H) domain. The RT and RNase H domains have sequences highly conserved among proteins with similar enzymatic functions such as the HIV RT[4,5]. Similar to the HIV RT, HBV RT lacks proofreading activity[6,7]. As a result, HBV exhibits an estimated mutation rate of 1.4×10^{-5}-3.2×10^{-5} nucleotides per site per year, which is more than 10-fold higher than other DNA viruses[8-10]. The high error rate of HBV RT causes frequent nucleotide substitutions during viral replication, resulting in genetic diversity in the form of genotypes, sub-genotypes, quasispecies and a large number of mutations in different regions of the HBV genome. With a spontaneous error rate of 10^{-5} substitution/base/cycle, viral mutants are generated every day in a mixture of viral quasispecies within the same patient. These mutants usually confer disadvantages to the replication, assembly, secretion or infectivity of the virus; however, in the context of pressure due to antiviral immune response or therapy, the mutants can be selected and become the dominant species.

The natural history of HBV infection can vary dramatically depending on both host and viral factors. Most people do not experience any symptoms during the acute infection phase. However, some people have acute illness with symptoms that last several weeks. A small subset of people with acute hepatitis can develop acute liver failure which can lead to death. Acute HBV infection is characterized by the presence of HBsAg and immunoglobulin M antibody to the core antigen, HBcAg. During the initial phase of infection, patients are also seropositive for HBeAg. HBeAg is usually a marker of high levels of virus replication. The presence of HBeAg indicates that the blood and body fluids of the infected individual are highly contagious. In adults, about 5% of otherwise healthy persons who are infected with HBV as adults will develop chronic infection. Chronic infection is characterized by the persistence of HBsAg for at least 6 mo (with or without concurrent HBeAg). Persistence of HBsAg is the principal marker of risk for developing chronic liver disease and HCC. The 20%-30% of adults who are chronically infected will develop cirrhosis and/or hepatocellular carcinoma (HCC). Some people can develop occult HBV infection. Occult HBV infection is defined as the presence of HBV DNA in the liver tissue of HBsAg-negative individuals[11,12].

Abundant evidence has shown that the genetic diversity of HBV plays critical roles in modulating the pathogenesis in HBV infection (Figure 1). HBV genotypes, sub-genotypes and mutations in certain regions of the HBV genome have been found to influence the HBeAg seroconversion rates, HBcAg seroconversion, viremia levels, immune escape, emergence of mutants, pathogenesis of liver disease, response and resistance to antiviral therapy, and vaccination against the virus (Figure 2).

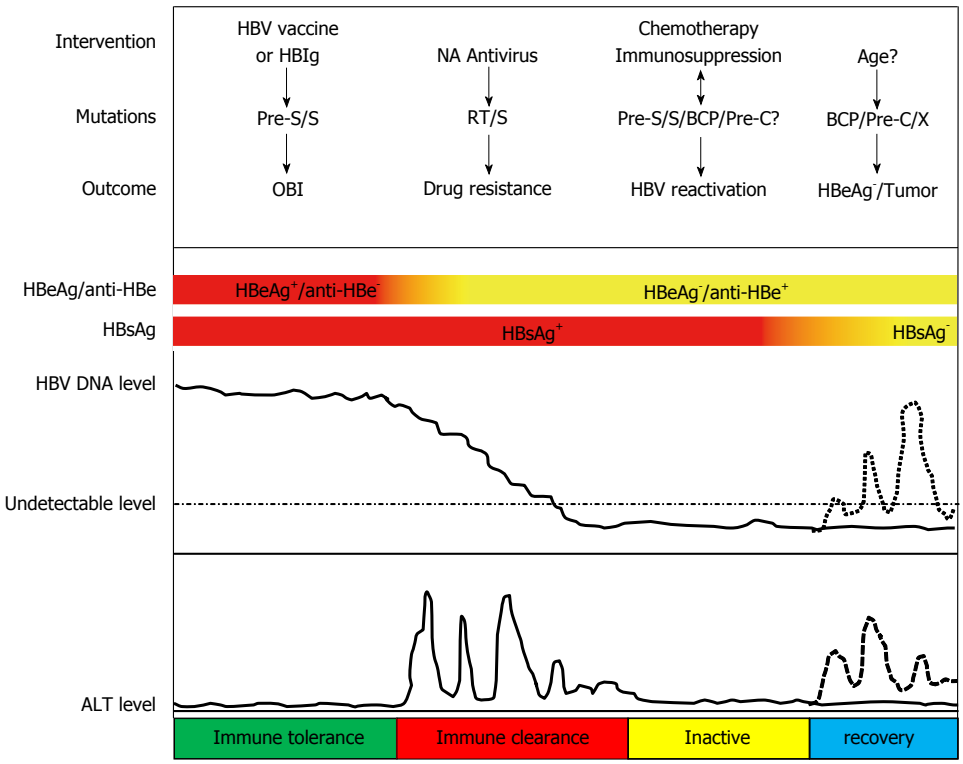

Figure 1 Interventions, mutations and clinical outcomes in the natural history of hepatitis B virus infection. The natural history of hepatitis B virus (HBV) infection is typically classified into 4 phases: immune tolerant, immune clearance, inactive and recovery phase. Patients who receive HBV vaccine and/or hepatitis B immunoglobulin (HBIg) injection may develop chronic HBV infection with PreS and/or S gene mutations, this phenomenon is called occult HBV infection (OBI); during the immune clearance phase, long-term use of NAs can select for mutations in the reverse transcriptase (RT) and S regions; HBV reactivation may occur during the inactive phase in patients with hepatitis B surface antigens (HBsAg) and/or anti-HBc positive after chemotherapy or immunosuppression. HBV isolated from these patients may harbor mutations in the PreS, S, basal core promoter (BCP) or Pre-C regions. The causal relationship between these mutations and chemotherapy or immunosuppression is unclear; with increasing age, some patients can develop BCP, Pre-C or X region mutations or HBV DNA integration leading to hepatitis B e antigen (HBeAg) negativity or tumor.

GENOTYPES AND SUB-GENOTYPES

HBV was formerly classified into nine serological subtypes according to the antigenic determinants[13]. In 1988, Okamoto *et al*[14] compared the full nucleotide sequence of 18 HBV strains and classified them into four groups, genotype A to D, by a divergence of more than 8% between genotypes. Since then, at least 10 genotypes (A to J) have been identified. By a divergence of 4%, HBV genotypes can be further classified into sub-genotypes. This approach has resulted in HBV genotype A (A1-A7), genotype B (B1-B9), genotype C (C1-16), genotype D (D1-D8), and genotype F (F1-F4)[15-17]. Similar to HBV serological subtypes, HBV genotypes and sub-genotypes also have distinct geographical distributions. HBV sub-genotype B1 dominates in Japan, B2 dominates in China and Vietnam, B3 is confined to Indonesia, and B4 is confined to Vietnam[18]. B7, B8, and B9 have been found in an island in Southeast Asia[19]. HBV/C1 (Cs) is found mainly in Southeast Asia, whereas C2 (Ce) is predominant in East Asia[20]. HBV/C3 was confined to Oceania, while C4 (Caus) was exclusively found in Australia and regarded as the most divergent sub-genotype within HBV/C[21]. Sub-genotypes C5 and C7 were found in Philippines, while C6 and C8 to C16 were isolated from Indonesia[22-27]. This pattern of defined geographical distribution was less evident for D1-D4, where the sub-genotypes were widely spread in Europe, Africa, and Asia[18]. Moreover, as was pointed out in a recent review article, immigration has become an important confounding factor of global HBV distribution and has been substantially changing the geographic pattern of HBV sub-genotypes[28]. Notably, the intergenotype recombination has also been described previously, which plays an important role in the evolutionary history of HBV. Recombination is favored in particular geographical regions[29,30]. For instance, B/C recombinants are prevalent in Southeast Asia and East Asia[31]. Other intergenotype recombinants such as A/D, A/E, C/D and G/C recombinants have also been observed in different geographical regions[29-31]. Therefore, it is logical to predict that distinguishing exotic (sub)genotypes from native ones will become more and more important for improved prophylaxis, diagnosis and treatment.

Not only are HBV genotypes and sub-genotypes related to geographical distribution, mounting evidence has shown that they are also associated with the pathogenesis and outcome of HBV infection. An early study from Europe found that genotype A infection was associated with a significantly higher rate of

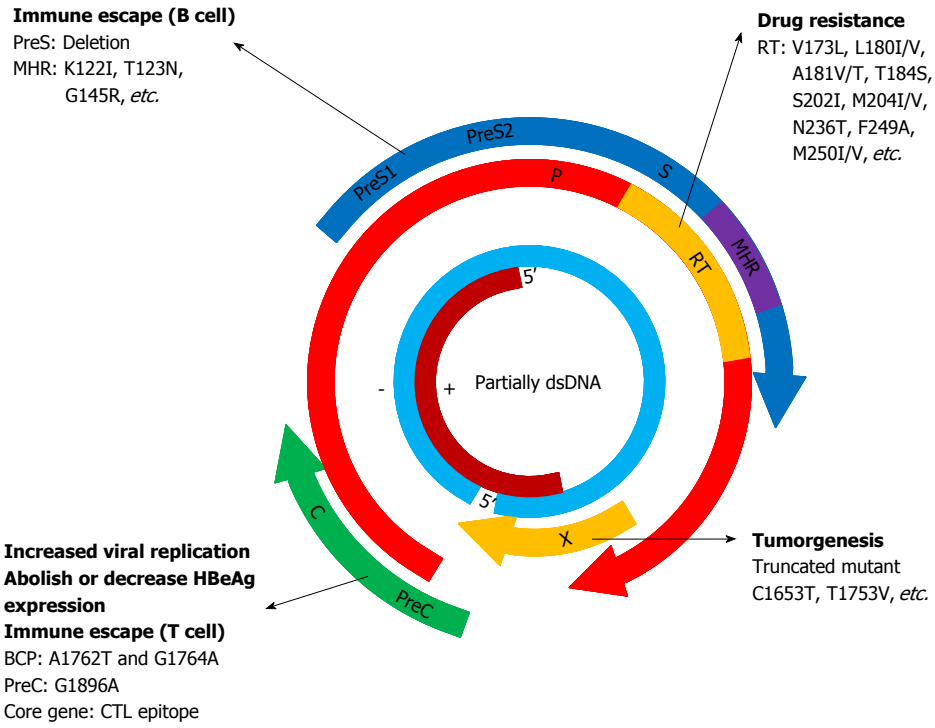

Figure 2 Hepatitis B virus genome and the major gene mutation types in hepatitis B virus open reading frames. The hepatitis B virus (HBV) genome is a 3.2 kb double-stranded DNA molecule that is organized into four open reading frames: the polymerase, the envelope, the precore and X. The deletion mutations in the PreS gene region and/or some point mutations in the major hydrophilic region (MHR) of S gene can lead to immune escape and occult HBV infection; mutations in reverse transcriptase (RT) can lead to drug resistance after long-term use of nucleotide analogues and this drug-resistant HBV typically has an altered viral envelope of hepatitis B surface antigen because of the overlap between the polymerase and envelope, for example, A181T/V mutations in the RT region can cause W172* (stop codon mutation), W172L and L173F mutations in the S region. M204V/I mutations in the RT region can result in I195M, W196* (stop codon mutation), W196S and W196L mutations in the S region; A1762T and G1764A mutations in the base core promotor (BCP) or G1896A mutation in PreC can increased viral replication, abolish or decrease HBeAg expression. Some mutations in the CTL epitope of HBV core gene can cause T cell immune escape; some point mutations or truncated mutants in the HBV X gene can cause tumorigenesis or other end-stage liver disease.

sustained biochemical remission, HBV DNA clearance, and HBsAg clearance in patients with chronic HBV infection than genotype D infection[32]. Similarly, a study from China has also shown that genotype A and B patients have a higher rate of HBsAg sero-clearance than genotype C and D patients[33]. A study from India has also suggested that genotype D was associated with more severe liver diseases and HCC in younger patients than genotype A[34]. Furthermore, a study from Alaska, where five of the ten HBV genotypes are present, showed that the rate of complications, including HCC, for those infected with genotype A appeared to be less than that found in individuals infected with genotype D, C, or F1[35]. A study by the same group also revealed that HBeAg seroconversion occurred about 3 decades later in women infected with genotype C than those infected with genotypes A2, B6, D, and F1[36]. Multiple cross-sectional studies have suggested that patients with genotype C experience HBeAg seroconversion at older ages and are more likely to be HBeAg positive at any given age than HBV genotype B[37-39]. HBV genotype C has also been associated with increased risk of liver inflammation, flares of hepatitis, liver fibrosis, and cirrhosis[37,38,40]. Prospective studies compared the outcome in those infected with genotypes B and C further and have also shown that HBeAg seroconversion occurred at a significantly younger age for those infected with genotype B than genotype C[36,41-43], and that increased risk of fibrosis was associated with genotype C[42,44]. In addition, infection with genotype C has been identified as an independent risk factor for the development of HCC[45]. Taken together, it can be concluded that individuals infected with HBV genotype C seroconvert from HBeAg later in life and have an increased risk of liver inflammation, liver fibrosis, and HCC. In an acute liver failure study in the United States, genotype D was found to be an independent risk factor for fulminant hepatitis[46]. Genotype G is the most uncommon of all HBV genotypes. This genotype is almost exclusively found in persons co-infected with another HBV genotype, most commonly genotype A, with the only exception being a single report of a transfusion-associated case[47,48]. HBV genotypes F and H are the "New World" genotypes found primarily in indigenous populations of North and South America. In a nested case-control study of a cohort of 1176 Alaskan Natives with chronic HBV infection followed up for 20 years, a significantly higher proportion of persons infected with either genotype F1 or genotype C2 developed HCC than those infected with genotype A2, B6, or D[35]. Genotype H is most closely related to genotype F and

likely evolved from this genotype.

The current definitive method for HBV genotyping is PCR amplification and sequencing of the entire genome followed by phylogenetic analysis[14,49-51]. Studies on HBV concerning different genotypes frequently face problems regarding representativeness of reference strains. Taking advantage of large number of sequences deposited in the GenBank database, we have developed a strategy to establish reference sequences for different genotypes. Briefly, sequences were clustered and genotyped using phylogenetic analyses first, and sequences belonging to the same genotype were then aligned with each other and the most common nucleotides in each position were chosen to form the reference sequence for this particular genotype[52]. Although HBV consensus sequences can be generated by sequence alignment, they may not exist in nature or can not usually be isolated from patient samples. To solve this problem, we have adopted a chemical synthesis strategy to generate the consensus HBV genome for certain genotypes. In our recent study, genotype B consensus sequence was established by comparing 42 full-length HBV genotype B sequences and the genome was generated by chemical synthesis. A plasmid carrying a 1.3 × full-length chemically synthesized HBV consensus genome was constructed. This consensus genome was fully replication competent when transfected into hepatoma cells. After this plasmid was hydrodynamically injected into BALB/c mice, HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc and HBV genome replication were all detected. Thus, this approach represents a novel strategy to design and create HBV genomes for future studies[53].

PRE-S/S MUTATIONS

The preS/S ORF encodes proteins L, M and S. The Pre-S1 domain is unique for the L protein. The Pre-S2 domain is the shared sequence with the M protein and the S domain is seen in all three proteins. The L and S proteins are essential for virion formation and the M protein can increase the efficiency of virion secretion[8,54,55]. The dominant epitopes of HBsAg, which are the targets of neutralizing B cell responses, are located in the "α" determinant (aa 124-147) within the HBsAg major hydrophilic region (MHR), which covers aa 99-169[56-62]. Mutations in the MHR, particularly the "a" determinant, are known to be associated with immune escape due to conformational changes in the epitope resulting in reduced binding affinity between the HBsAg and the antibody to HBsAg. Carman *et al*[56] first reported the G145R mutation in the "a" determinant of HBsAg in a child who became infected with HBV despite active and passive immunoprophylaxis. Subsequently, several other mutations within and outside the "α" determinant were reported to have reduced binding affinity to anti-HBs[57,63,64]. To date, escape mutations in the HBsAg MHR have been comprehensively analyzed[65-67].

In 2003, we noted that a renal transplant recipient was persistently positive for HBV despite preexisting anti-hepatitis B surface antibodies (anti-HBs). We cloned the HBsAg gene that was found to be mutated from the patient and compared the antigenicity and immunogenicity of the mutant HBsAg with wild-type HBsAg (wtHBsAg) in mice using a genetic vaccination approach. Our results showed that both B cell and T cell immune responses were impaired by these mutations, suggesting that mutations within HBsAg may enable HBV to escape immunological control[68]. Subsequently, in an agenetic vaccination animal model, we demonstrated that serum samples from mice immunized with aa substitution G145R could recognize plasma-derived mtHBsAg, suggesting HBsAg with aa substitutions may be immunogenic, but with changed specificity[69]. We further investigated the impact of some naturally occurring HBsAg mutations around position 120 to 123 on the antigenicity and detection of HBsAg. Strikingly, aa substitution K122I abolished the reactivity of HBsAg in all immunoassays tested. mtHBsAg G145R was clearly detected with four different enzyme-linked immunosorbent assays that were based on monoclonal anti-HBs antibodies (MAbs) with high affinity. Positive immunofluorescence staining of mtHBsAg K122I was achieved only by polyclonal anti-HBs, while all tests using MAbs failed. mtHBsAg T123N showed a low reactivity in immunoassays and appeared to be secretion deficient. The aa substitution P120T reduced the binding of anti-HBs, but did not completely prevent the detection of mtHBsAg by anti-HBs MAbs. Thus, our data showed that the presence of aa substitutions within the region of 120 to 123 is essential for HBsAg antigenicity and strongly associated with impaired detection in immunoassays[70]. In another study, we systematically investigated a variety of aa substitutions that have been identified around or within the "α" determinant of HBsAg, such as K122I and G145R, on HBsAg expression, secretion and antibody binding. The results showed that the hydrophobicity, the presence of the phenyl group, and the charges in the side chain of the aa residues at position 145 reduced HBsAg secretion and impaired reactivity with anti-HBs antibodies. Only the substitution K122I at position 122 affected HBsAg secretion and recognition by anti-HBs antibodies. Genetic immunization in mice further demonstrated that the priming of anti-HBs antibody response was strongly impaired by the substitutions K122I, G145R, and others, such as G145I, G145W, and G145E. Moreover, when mice that had been pre-immunized with wtHBsAg or variant HBsAg (vtHBsAg) were challenged by hydrodynamic injection (HI) with a replication-competent HBV clone, HBsAg persisted in peripheral blood for at least 3 d after HI in mice pre-immunized with vtHBsAg, but was undetectable in mice pre-immunized with wtHBsAg, indicating that vtHBsAgs fail to induce proper immune responses for

efficient HBsAg clearance. Therefore, the biochemical properties of aa residues at positions 122 and 145 of HBsAg have a major effect on antigenicity and immunogenicity. In addition, the presence of proper anti-HBs antibodies is essential for the neutralization and clearance of HBsAg during HBV infection[71].

Viral envelope N-glycosylation modification has been known to play important roles in the biogenesis, stability, antigenicity and infectivity in HIV, HCV and influenza virus[72,73]. Previously, a few studies indicated that a potential N-glycosylation site (Asn-X-Ser/Thr, where X is any aa except Pro) could be created by some mutations within the HBsAg MHR[62,74]. In a recent study, we systematically examined the effects of naturally occurring N-glycosylation-related aa substitutions K122I, T123N, A159G and K160N. T123N and K160N substitutions resulted in additional N-glycosylated forms of HBsAg, while the other mutations produced more heavily glycosylated HBsAg compared with the wild-type (wt). These results showed that vtHBsAg with K122I could not be recognized by HBsAg immunoassays, ELISA or immunofluorescence staining, vtHBsAg with T123N, A159G, K160N and A159G/K160N could be detected, but showed reduced antigenicity. DNA immunization in BALB/c mice revealed that wtHBsAg and vtHBsAg with T123N and K160N were able to induce antibodies to HBsAg (anti-HBs), whereas K122I and A159G greatly impaired the ability of HBsAg to trigger anti-HBs responses. The cellular immune response to the HBsAg aa 29-38 epitope was enhanced by the K160N substitution. Replication competent clones of HBV, T123N and A159G substitutions were shown to strongly reduce virion assembly. The aa substitution K160N appeared to compensate for the negative effect of A159G on virion production. Thus, our study revealed complex effects of aa substitutions on the biochemical properties of HBsAg, on antigenicity and immunogenicity, and on the replication of HBV[75]. Another recent study investigated the molecular and clinical characteristics of HBV immune escape mutants in a Chinese cohort of chronically infected patients. By investigating 216 patients with double positive HBsAg and anti-HBs and 182 HBV carriers without anti-HBs as a control group, the authors found that the frequency of N-glycosylation mutations in the patient group was much higher than that in the control group (47/216 *vs* 1/182). Using a chemiluminescent microparticle enzyme immunoassay, they showed that HBsAg mutants reacted weakly with anti-HBs compared with wtHBsAg. Their native gel analysis of secreted virion in supernatants of transfected Huh7 cells further revealed that mutants had better virion envelopment and secretion capacity than wt HBV[76]. Together these studies strongly suggested that N-glycosylation mutations on HBsAg greatly contribute to immune escape.

Numerous studies including ours have demonstrated that immune escape mutations were the underlying mechanisms for HBsAg and anti-HBs double positive status[60,77,78]. These mutations are also responsible for a large portion of failure in immunoprophylaxis. In Taiwan, the prevalence of surface gene mutants was found to be approximately 20% in HBsAg carrier children[79]. A study from China reported that the prevalence of aa substitutions among immunoprophylaxis failed children was 20%[80]. Another study reported that the nucleotide diversity rate was 11.4% in those children in Jiangsu Province[81]. The mutation rate in children who failed to be protected by perinatal prophylaxis in Singapore, Japan, and the United States has been reported to be 39.0%, 30.8% and 25.5%, respectively[64,82,83].

Variants within the MHR of HBsAg have also been associated with occult HBV infection (OBI). A study from China by Hou *et al*[84] found that in 46 cases of OBI, 32 aa substitutions were found between positions 100-160 within the MHR. In addition to the G145R, 11 positions inside and 5 positions outside the "α" determinant were involved. Combined mutations were also detected in some patients. Another two patients had insertion mutations immediately before the "α" determinant. Another study conducted in China in recent years identified another 8 escape mutations associated with OBI, in addition to the G145R, mainly located at positions 120, 126, 129, 130, 133, 134, 137, 140, 143 and 144[66]. In a recent cohort study conducted in South Korea, mutations in the "α" determinant were also found to be related to OBI[85]. Another recent study from China compared the characteristics of 61 patients with OBI to 153 HBsAg (+) carriers with low titers of serum HBsAg (HBsAg-L group) and 54 samples with high serum HBsAg (HBsAg-H group). MHR mutations were seen significantly more frequently in OBI cases (55.7%) compared to the HBsAg-L (34.0%) or the HBsAg-H groups (17.1%). 13 representative MHR mutations were observed in patients with OBI. Of which, 4 mutations strongly decreased the analytical sensitivity of 7 commercial HBsAg immunoassays and 10 mutations significantly impaired virion and/or S protein secretion in both Huh7 cells and mice[86].

Mutations in the pre-S region, especially deletions, have been associated with a lack of detectable HBsAg in the serum. Deletions in the pre-S region can result in reduced expression of HBV surface proteins and help viral persistence by eliminating HLA-restricted B-cell and T-cell epitopes. Pre-S1/pre-S2 mutations are also frequently detected in OBI[85,87,88]. In one study, a 183-bp deletion (nt 3019 to 3201) in the pre-S1 region was detected in occult HBV patients. The deletion covered the CCAAT element that is required for transcription factor binding. The association of deletions in the *pre-S* gene with a lack of secreted HBsAg was demonstrated using functional analysis by transfection into hepatocyte cell lines[89]. In a similar study, Xu *et al*[90] showed that a 129-bp in-frame deletion in the S promoter region was associated with reduced levels of M and S protein transcripts, resulting in a marked reduction in the expression of

the two proteins. The roles of S promoter mutations and deletions in HBsAg production and secretion in OBI have also been reported in other studies[87,91]. In a recent study, a male patient from China with a chronic hepatitis B infection for 30 years was diagnosed with HB-LF. We amplified the HBV sequences from this patient and found that 2 major variants coexisted in the ratio of 1 to 4: the first variant harbored the A1762T/G1764A 257 double mutation in the basal core promoter (BCP) region and the G1896A mutation in the preC region; the second variant contained 2 deletions in the preS1 region (nt 2976-3102) and the preS2 promoter and preS2 ORF region (nt 3203-3215, nt 1-31), resulting in a stop codon mutation in the ORF of large HBsAg and the deletion of the start codon in the ORF of middle HBsAg, respectively. When we transfected replication competent plasmids harboring these variants into Huh7 cells, we observed phenotypes one would normally predict. However, when both constructs were co-transfected into Huh7 cells, new phenotypes arose. For example, coexistence of both variants increased HBV replication and led to the predominant nuclear localization of HBcAg. Moreover, mice mounted significantly stronger antibody and cytotoxic T lymphocyte (CTL) responses to HBsAg when both variants were co-applied in the HI mouse model. Thus, the coexistence of preS deletion mutants with other variants may significantly modulate specific host immune responses and may enhance immune-mediated liver damage under some circumstances, which represents a novel mechanism for the immunopathogenesis of HBV infection[92].

Numerous studies have linked preS, especially preS2 deletions to the occurrence of HCC[93-98]. At least three mechanisms have been suggested to be involved in the pathogenesis of preS deletion-associated HCC: Firstly, the preS1 and preS2 regions contain several epitopes for T and B cells, and play essential roles in the interaction with host immune responses. Therefore, preS deletion mutations may result in an inefficient immune response[99,100]. Secondly, pre-S1 and pre-S2 mutations may cause overproduction and accumulation of L protein in the endoplasmic reticulum (ER), resulting in significant ER stress that may induce DNA damage and genomic instability leading to hepatocarcinogenesis[101]. Thirdly, preS2 mutants have been shown to directly activate tumor promoting pathways such as the VEGF/AKT/mTOR pathway and the p27/retinoblastoma/Cdk2/cyclin A, D pathway, among others[102-106]. In fact, the pre-S mutants have been shown to be capable of inducing dysplasia in hepatocytes and the development of HCC in transgenic mice[107].

S region mutations have also been associated with acute exacerbation of liver diseases leading to fatal liver failure. In our study, a Chinese patient who suffered from acute liver failure after discontinuation of lamivudine treatment was described. The patient was treated with lamivudine for 4 mo and ceased treatment without consulting. After receiving lamivudine, the patient developed anti-HBs and became negative for hepatitis B surface antigens (HBsAg). However, the patient suffered from a severe exacerbation about two months after cessation of treatment and died due to acute liver failure. Sequencing of HBV isolates revealed mutations including G145R and stop codon mutations at the aa positions 74 and 199 in the HBsAg sequences in all clones. F134S and F134V substitutions within the a-determinant were also detected in some clones. Various aa substitutions were present outside the a-determinant. No wt clone was found among the six cloned sequences. Importantly, no lamivudine resistance-associated mutation was found in the RT region coding for the HBV polymerase protein. The data suggested that anti-HBs antibody which appeared during the lamivudine treatment might be the selective force for the emergence of HBV mutants, and HBV replication resumed after the cessation of lamivudine treatment in this patient might have triggered the process leading to liver failure[108].

In a recently published study, a correlation was revealed between some HBsAg-mutations and serum HBV-DNA levels in HBV chronically-infected drug-naive patients. In this study, 187 patients were stratified into the following ranges of serum HBV-DNA: 12-2000 IU/mL, 2000-100000 IU/mL, and > 100000 IU/mL. The S gene of HBV was isolated and sequenced. Mutant and wt HBV genomes were expressed in Huh7 cells and HBsAg production was determined in cell-supernatants 3 d post-transfection. The results showed that HBsAg-mutations M197T, S204N, Y206C/H and F220L were significantly correlated with serum HBV-DNA < 2000 IU/mL (posterior-probability > 90%, $P < 0.05$), and the presence of Y206C/H and/or F220L was also associated with lower median (IQR) HBsAg-levels and lower median (IQR) transaminases [for HBsAg: 250 (115-840) IU/mL for Y206C/H and/or F220L *vs* 4300 (640-11838) IU/mL for wt, $P = 0.023$; for ALT: 28 (21-40) IU/mL *vs* 53 (34-90) IU/mL, $P < 0.001$). These mutations were localized in the HBsAg C-terminus, known to be involved in virion and/or HBsAg secretion. Thus, specific HBsAg-mutations in the HBsAg C-terminus correlated with low-serum HBV-DNA and HBsAg-levels. These mutations may represent an important mechanism underlying low HBV replication and the inactive-carrier state[109].

P REGION MUTATIONS

The P ORF encodes the viral DNA polymerase protein P, which is a specialized RT. Except for interferon-α and pegylated interferon-α, all five drugs approved for HBV treatment are nucleos(t)ide analogues (NAs) that target the DNA polymerase activity of this protein. Treatment with these NAs is generally efficient and well tolerated. However, resistance to some of these agents is a major issue affecting long-term therapy. Lamivudine resistance occurs frequently

and is observed in up to 80% of patients treated for 5 years[110-112]. Among adefovir-treated patients, the cumulative incidence of resistance over 5 years has been reported to be 29% in HBeAg-negative patients and 42% in HBeAg-positive patients[113,114]. With 25% of HBeAg-positive and 11% of HBeAg-negative patients experiencing virological breakthrough due to resistance after 2 years of treatment, telbivudine resistance is relatively slower to emerge[115]. Resistance to entecavir has been shown to remain low (1.2%) after 6 years of therapy in NA-naıve patients[116]. No tenofovir resistance has been observed after 4 years of treatment in the registration studies[117].

Antiviral drug resistance is associated with the selection of adaptive mutations which reduces the sensitivity of the mutants to the inhibitory effects of a drug. The barrier to resistance can be defined as the difficulty with which the resistance mutants are selected and the barrier to resistance increases as the number of specific mutations required for drug resistance increase[118]. The main aa change associated with lamivudine and telbivudine-resistance is rtM204V/I, which is located in the YMDD motif of the RT. In the context of lamivudine resistance, the compensatory mutations rtL180M and rtV173L frequently occur in domain B and C of the viral polymerase[4]. The rtA181V/T, rtL80I/V and rtM204Q mutants identified recently showed that primary lamivudine-resistance mutations can also occur outside the YMDD motif[119,120]. Lamivudine-resistance mutations have been reported to result in the selection of high replicative HBV variants leading to exacerbation of disease during chronic HBV infections. In an early study, full-length HBV genomes were analyzed from four chronic hepatitis B patients who developed resistance to lamivudine [-2'-deoxy-3'-thiacytidine, LMV] accompanied by acute exacerbation of disease. Paired full-length HBV isolates were cloned from the sera of patients prior to LMV treatment and after drug resistant breakthrough. Compared to the isolates before treatment, isolates from all four patients during exacerbation showed a marked increase in replicative competence in a cell transfection study. Viral genome amplification and sequencing showed that while all isolates shared mutations at the YMDD motif, the isolates from the one patient who recovered from the exacerbation showed a lower number of mutations, and in particular, lacked BCP mutations at 1762/1764. In contrast, BCP mutations were found in isolates from the other three patients. Thus, in patients with acute exacerbation, high replicative strains were selected from the total HBV quasispecies during treatment, and among these strains, those with core promoter mutations at 1762/1764 were most likely to be associated with severe clinical exacerbations[121]. Adefovir resistance is characterized by rtN236T and/or rt181V/T selected in the D and B domain of the polymerase, respectively[122-124]. The rtI233V mutation was found to confer resistance to adefovir[124]. Entecavir is a NA with a high barrier of resistance as multiple mutations are required to confer a high level of resistance to this drug. Entecavir resistance tends to emerge in a stepwise manner with the rtI169T, rtT184S, rtS202I, and rtM250I/V changes occurring sequentially in the virus already carrying lamivudine resistance mutations[125,126].

No tenofovir resistance has been described after 3 and 4 years of therapy, but rt181T/V and rtN236T, the main adefovir-associated resistance mutations, do reduce its sensitivity clinically and virologically[117,122,127]. Tenofovir (TDF) has been a first-line antiretroviral drug for human immunodeficiency virus (HIV) since 2001 and it is well known that this drug induces resistance mutations leading to treatment failure. Taking advantages of the high homology between the HIV and HBV RTs and the determination of the crystal structure of HIV-1 RT complexed with TDF, we built the homology model for HBV-RT[5,128]. Based on the modeled HBV-RT structure, we designed some mutants and tested their effects on TDF susceptibility *in vitro* in Huh7 cells and *in vivo* in a mouse model. Our results showed that HBV mutants with rtP177G and rtF249A reduced susceptibility to tenofovir *in vitro* with a resistance index of 2.53 and 12.16, respectively, and the testing result based on the HI mouse model revealed the antiviral effect of TDF against wt and mutated HBV genomes, and confirmed the reduced susceptibility of mutant HBV to TDF[129]. In a recent population-based cross-sectional study from China, serum samples from 179 patients who developed virological breakthrough while receiving treatment with NAs were obtained and analyzed for NA-resistant mutations in the RT region[130]. NA-resistant mutations were detected in 89.4% (160/179) of these patients. The prevalence of HBV mutations at rtM204 was 93.0% (106/114) in patients on lamivudine/telbivudine-based therapy, with rtM204I being more frequently associated with rtL80I/V mutations [rtM204I + rtL80I/V (50.0%, 32/64) *vs* rtM204V + rtL80I/V (27.3%, 9/33), $P = 0.032$]; rtN236 mutations were found in 76.1% (35/46) of patients receiving adefovir/dipivoxil-based therapies, with rtM204V mutations being more frequently associated with the rtL180M mutations [rtM204V + rtL180M (100%, 33/33) *vs* rtM204I + rtL180M (60.9%, 39/64), $P < 0.001$]. In addition, rtA181 mutations were observed in 19.3% (22/114) of patients receiving lamivudine/telbivudine-based therapy and 23.9% (11/46) of patients receiving adefovir/dipivoxil-based therapy.

The RT and HBsAg ORFs overlap at RT aa 8-236, and the HBsAg ORF shifts downstream by 1 nucleotide. Not surprisingly, some resistance mutations also affect the overlapping HBsAg. For instance, the rtA181T mutant selected by adefovir, lamivudine or telbivudine typically results in a stop codon in the envelope gene (sW172stop), causing a dominant negative secretion defect in HBsAg leading to an altered viral rebound profile[131]. Studies have also shown that common

antiviral drug selected mutations may confer changes in the antigenicity of the overlapping HBsAg. A pioneering study by Torresi *et al*[132] reported that resistance mutations rtV173L + rtL180M + rtM204V that resulted in mutations sE164D + sI195M in HBsAg reduced antigen-antibody binding. A few years later, Sloan *et al*[133] confirmed their observations and further revealed that these mutations caused immune evasion through disruption of the "α" determinant on HBsAg. In recent years, similar observations have been made in studies from Brazil and China[134,135]. On the other hand, the overlapping changes in surface genes may potentially affect the impact of HBV polymerase mutation on replication and drug resistance. For example, the sW172 stop mutation could result in decreased viral replication and increased drug resistance[136,137]. Additionally, wt HBV and drug-resistant HBV such as the sW172 stop mutation could complement each other to maintain viral replication and rescue virion production under NAs treatment, thus facilitating HBV survival and persistence under NAs pressure (our unpublished data).

C REGION

The HBV core gene is divided into the precore (PC) region and the basic core region (BCP) by two in-frame initiating ATG codons. This results in the transcription of either the pregenomic RNA that is essential for HBV replication and translates into the nucleocapsid protein HBcAg or the PC RNA that translates into HBV e antigen (HBeAg) protein. Studies have linked defect core protein expression to PC and/or BCP mutations. The most prevalent PC mutation is a guanine-to-adenine transition at nucleotide position 1896 (G1896A), which creates a TAG stop codon at codon 28 of the PC protein and abolishes HBeAg expression at the translational level[138]. The most common BCP mutation is the double A1762T and G1764A nucleotide exchange, which results in a decrease in HBeAg expression of up to 70%, but enhanced viral genome replication[138-140].

The core protein HBcAg of HBV is a potent immune stimulator, stimulating a strong neutralizing immune response[141,142]. Cytotoxic T cells (CTLs) play a key role in the control of HBV infection and viral clearance. The HBV-specific CD8+ T lymphocytes (CTL)-mediated immune response is multi-specific, polyclonal, and vigorous during acute hepatitis B (AHB), which plays a vital role in viral control and viral clearance, as well as disease pathogenesis[143-145]. In contrast, the HBV-specific CTL response is minimal or undetectable in chronic hepatitis B (CHB) with viral persistence and immune tolerance, indicating the key role of HBV-specific T-cell response in the determination of disease progression and outcome[146,147]. Analysis of CTLs specific for viral epitopes within core, envelope, polymerase, and X proteins showed that the highly conserved HBV core protein (HBc) elicits the strongest CTL responses compared with other viral proteins, suggesting that the HBc-specific T cell response may play a leading role in viral control and clearance[148-152]. However, mutations in HBcAg may lead to the production of immune escape variants, resulting in the persistence of HBV[2,153]. In a recent study, the HBV core gene was amplified and sequenced from 148 patients with chronic HBV infection, and the human leukocyte antigen (HLA) class Ⅰ genotype (A and B loci) of the patients was determined. Using a statistical approach with a novel analysis package SeqFeatR, residues under selection pressure in the presence of particular HLA class I alleles were identified. With this approach, nine residues in HBV core under selection pressure in the presence of 10 different HLA class I alleles were identified. Immunological experiments confirmed that seven of these residues were located inside epitopes targeted by patients with chronic HBV infection carrying the relevant HLA class I allele. Consistent with viral escape, the selected substitutions reproducibly impaired recognition by HBV-specific CD8 T cells[154].

HBeAg is not required for HBV replication *in vitro*, but is secreted into the blood. Acting as both an immunogen and a tolerogen, it has profound effects on the natural history and pathogenesis of HBV infection[155]. In general, HBV replicates more actively in carriers with HBeAg than those with anti-HBe, and carriers with HBeAg have a higher activity to transmit HBV than those with anti-HBe[156]. In chronic hepatitis B infection, a key event in the natural history of progression is HBeAg seroconversion to HBeAb with a marked reduction of HBV replication followed by gradual histological improvement[157]. However, a proportion of patients who undergo HBeAg seroconversion demonstrate a recurrence of high HBV DNA levels and intermittent or persistent ALT level elevations. These individuals harbor a mutant form of HBV that does not produce HBeAg, due to a mutation in the precore or core promoter region. In Asia, the Middle East, Mediterranean basin and southern Europe, about 15% to 20% of these carriers have elevated alanine aminotransferase and viral DNA[158]. HBeAg-negative chronic hepatitis B (precore mutant) emerges as the predominant species during the course of typical HBV infection with wt virus and is selected during the immune clearance phase (HBeAg seroconversion)[159]. Sustained spontaneous remission is rare (6% to 15%) in these individuals, and spontaneous HBsAg clearance is only about 0.5% per year[160]. Therefore, long-term prognosis is poorer among HBeAg-negative individuals than their HBeAg-positive counterparts. In fact, HBeAg-negative chronic hepatitis B is currently the main type worldwide as well as the most difficult to treat in terms of achieving sustained virological response.

In China, hepatitis B-related ACLF (HB-ACLF) patients account for more than 80% of ACLF cases as a result of the high incidence of chronic HBV

infection, with a high mortality rate of 60%-80% in the absence of liver transplantation, causing 22600 deaths annually[161,162]. Characterized by increased viral load and a fierce immune response, HB-ACLF is very often associated with mutations in the BCP and PC regions, because the BCP mutations may enhance HBV replication and the PC mutation abrogates translation of HBeAg, which is considered a tolerogen and immune repressor buffering the immune attack on the infected hepatocyte[163-168]. In fact, a higher prevalence of the BCP double mutation A1762T/G1764A and the G1896A PC mutation have been reported in ALF than in acute hepatitis B patients[169-173]. In addition, single mutations including the T1753V (C/A/G), C1766T, T1768A, G1862T and G1899A in the BCP/PC region have been reported to be associated with increased HBV replication capacity and/or reduced HBeAg expression *in vitro*, and in some cases associated with ALF in the clinic[46,163,173-176]. A recent study reported that T1846 and A/G1913 mutations are associated with ACLF in patients infected with HBV genotypes B and C[177].

X REGION

Chronic HBV infection is the dominant global cause of HCC, accounting for 55% of cases worldwide and 80% or more in the eastern Pacific region and sub-Saharan Africa[178]. Accumulating evidence has shown that HBxAg, the viral product of the X ORF, plays critical roles in the pathogenesis of HCC[179]. HBxAg promotes carcinogenesis by interacting with cellular proteins resulting in dysregulation of multiple signaling pathways involved in cell cycle progression, cell growth and apoptosis. To date HBxAg has been found to interfere with cellular signaling pathways including Src, pRb/E2F, p53, NF-κB, PI3K, Jak1/STAT, ERK and PI3K/AKT and Wnt/β-catenin[180-187]. During the last decade or so, several studies have indicated that the C-end truncated X protein often occurs in patients with HCC[188-194]. Further investigations revealed that the truncated HBxAg lost the proapoptotic activity of the full length form and thus acquired stronger cellular transformation activity *in vitro* and tumor promoting activity *in vivo*[189]. A recent study reported that, relative to WTHBxAg, naturally occurring truncated mutant HBxΔ127 strongly enhanced cell proliferation and migration in HCC[195]. In addition to truncated HBxAg mutants, insertions in the HBx gene may play a pivotal role in hepatocarcinogenesis. A Korean cohort study showed that the prevalence of insertions was significantly higher in patients with severe liver disease, HCC, or cirrhosis of the liver compared to patients who were carriers or had chronic hepatitis. Four novel types of insertions including PKLL, GM, FFN, and tt, were observed in six patients, which were accompanied by double mutations in the BCP region[196]. Moreover, site mutations have also been associated with HCC. Studies from Korea have reported that HCC risk increased in the presence of ⩾ 6 mutations of eight key mutations in Korean chronic HBV genotype C2 carriers. The eight key mutations comprise G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A and G1899A that are located throughout the core promoter and the proximal portion of the precore gene (X/preC region)[197,198]. A recent study assessed the postoperative prognostic value of HBV mutations in HBxAg in HBV associated HCC patients and found that eight mutational sites, including 1383, 1461, 1485, 1544, 1613, 1653, 1719, and 1753, could serve as independent predictors of HCC survival[199].

Mutations in reactivation of HBV infection upon chemotherapy and immunosuppression

Reactivation of HBV infection is a well-documented complication among cancer patients undergoing cytotoxic chemotherapy. In recent years, it has become clear that HBV mutations associated with severe liver diseases are frequently found in patients on chemotherapy. The most common mutations associated with chemotherapy-related HBV reactivation include the G to A mutation at nt 1896 in the preC/C region, the nt 1762 (A to T) and nt 1764 (G to A) mutations in the preC promoter region[200-204]. A study of ours suggested that immune escape mutations may also be involved in chemotherapy-associated HBV reactivation[205].

HBV reactivation can also occur during immunosuppression. A few years ago, we reported a case in which a non-Hodgkin lymphoma patient who had displayed positive anti-HBs and anti-HBc before immunosuppressive therapy developed HBV reactivation after receiving a rituximab-based regimen. Our sequencing data revealed genotype D with two known escape mutations P120S and S145P and three other mutations Y134K, I150T and T189I, which had not been found in the usual escape setting[206]. In a recent study, the genetic features of HBsAg were investigated by population-based and ultradeep sequencing (UDS) of HBV DNA from 93 patients: 29 developed HBV reactivation and 64 consecutive patients with chronic HBV infection (as controls)[207]. Of the HBV-reactivated patients, 51.7% were treated with rituximab, 34.5% with different chemotherapeutics, and 13.8% with corticosteroids only for inflammatory diseases. In total, 75.9% of HBV-reactivated patients (*vs* 3.1% of control patients; $P < 0.001$) carried HBsAg mutations localized in immune-active HBsAg regions. Of the 13 HBsAg mutations found in these patients, 8 of 13 (M103I-L109I-T118K-P120A-Y134H-S143L-D144E-S171F) reside in the MHR where neutralizing antibodies target. The remaining five (C48G-V96A-L175S-G185E-V190A) are localized in class Ⅰ/Ⅱ-restricted T-cell epitopes, suggesting a role in HBV escape from T-cell-mediated responses. Using UDS, these mutations occurred in HBV-reactivated patients with a median intra-patient

prevalence of 73.3% (range, 27.6%-100%) *vs* 4.6% (range, 2.5%-11.3%; $P < 0.001$) in control patients, supporting their fixation in the viral population as a predominant species. Moreover, additional N-linked glycosylation sites within the MHR were found in 24.1% of HBV-reactivated patients (*vs* 0% of chronic patients; $P < 0.001$). Thus, data from this study suggest that HBV reactivation occurs upon immunosuppression, correlating with HBsAg mutations endowed with enhanced capability to evade immune response. Another study cloned and sequenced the full length HBV genome from an HBsAg-negative patient who developed HBV reactivation following chemotherapy with rituximab[208]. The results showed that the number of aa substitutions in HBV from this patient was much higher than that reported for occult HBV infection or vaccine escape. It is worth noting that this study detected not only known "α" determinant mutations such as Q129H, F134Y, D144E and the preC G1896A mutation, but also a large number of mutations in other regions including preS, P, X and C, suggesting the possibility that mutations in other regions may also play some roles in immunosuppression-associated HBV reactivation.

Co-infection with HBV and human immunodeficiency virus (HIV) is not uncommon. It is estimated by the Joint United Nations Program on HIV/AIDS that 10% of 33 million HIV-infected patients has concurrent chronic HBV infection[209]. A higher proportion of chronic HBs antigenemia has been found in HIV-infected patients because HIV destroys CD4 cells which compromises host immunity against HBV[210]. Clinical observational studies have demonstrated that HIV/HBV-co-infected patients may have faster progression of hepatic fibrosis and a higher risk of cirrhosis, end-stage liver disease, and HCC than HBV-mono-infected patients[211,212]. The recurrence of HBV replication due to withdrawal of lamivudine therapy and administration of glucocorticosteroids in HIV/HBV-co-infected patients has been described previously[213-216]. Further observations including ours revealed that even slight suppression of host immunity by HIV infection at a level that did not require drug therapy could cause HBV reactivation[217,218]. It is worth noting that immune escape mutations were detected in most of these studies.

SUMARRY AND PERSPECTIVES

Most HBV genotypes and sub-genotypes have distinct geographical distributions. Abundant evidence has shown that genotypes and sub-genotypes are associated with the pathogenesis and outcome of HBV infection. Generally, HBV genotype C has been associated with an increased risk of liver inflammation, flares of hepatitis, liver fibrosis and HCC. Compared to other genotypes, patients with genotypes D, C, and F1 are more likely to develop complications such as liver cirrhosis and HCC. In addition, HBeAg seroconversion occurred much later in patients infected with genotype C compared to other genotypes. As shown in Figures 1 and 2, mutations in the preS/S region are associated with vaccine failure, immune escape, occult HBV infection and the occurrence of HCC. Mutations in the P region may cause drug resistance to NA antivirals. Mutations in the preC/C region are related to HBeAg negativity, immune escape, and persistent hepatitis. Mutations in the X region play critical roles in promoting HCC.

Investigations of HBV genetic variability and pathogenic implications of specific mutations have resulted in significant advances over the past decade. A significant increase in the body of knowledge regarding HBV genetic variability has greatly improved the way HBV infection is managed and treated. However, there are questions that remain unanswered and obstacles that need to be overcome. For example, much of our current understanding regarding HBV genetic variability was inferred from molecular epidemiological analyses. Due to constant viral evolution as a result of interactions among the host, virus and drug therapy, the results of these types of analyses can be confounded by many known or unknown factors. We believe that more physical experiments using reference strains that are really representative of their respective genotypes and sub-genotypes can help overcome this problem and provide more detailed and reliable information. In addition, as both viral and host factors affect HBV pathogenesis, reliable biomarkers and convenient methods need to be established to monitor both the viral and host factors to, ideally, achieve personalized management and treatment. As an example, in a recent pioneering study, Gong *et al*[219] compared the performance of next-generation sequencing and clone-based sequencing (CBS) in analyzing HBV RT quasispecies heterogeneity. In that study, HBV genomic DNA was extracted from serum samples obtained from 31 antiviral treatment-naive patients with chronic hepatitis B. The RT region quasispecies were analyzed in parallel using CBS and ultradeep pyrosequencing (UDPS). Their data showed that the number of qualified strains obtained by UDPS was much larger than that obtained by CBS ($P < 0.001$), and the complexity value derived from UDPS data was higher than that derived from CBS data ($P < 0.001$). A study on the prevalence of variations within the RT region showed that CBS detected an average of 9.7 ± 1.1 aa substitutions/sample and UDPS detected an average of 16.2 ± 1.4 aa substitutions/sample. This study clearly demonstrated that viral heterogeneity determination by the UDPS technique is more sensitive and efficient in detecting low-abundance variations than that by the CBS method, and thus has shed some light on the future clinical application of next generation sequencing in HBV quasispecies evaluation[219]. Additionally, it is important to continue research on the identification of novel therapeutic targets in the life cycle of HBV or in the host immune

system to stimulate the development of new antiviral agents and immunotherapies. These can be antiviral agents targeting HBV entry, cccDNA, capsid formation, viral morphogenesis and virion secretion, as well as therapeutic vaccines.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Overview of hepatitis B virus mutations and their implications in the management of infection

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Abstract

Hepatitis B virus (HBV) affects approximately two billion people worldwide and more than 240 million people in the world are currently chronic carrier that could develop serious complications in the future, like liver cirrhosis and hepatocellular carcinoma. Although an extended HBV immunization program is being carried out since the early '80s, representing effective preventive measure, leading to a dramatic reduction of HBV hepatitis incidence, globally HBV infection still represents a major public health problem. The HBV virus is a DNA virus belongs to the *Hepadnaviridae* family. The HBV-DNA is a circular, partial double strand genome. All coding information is on the minus DNA strand and it is organized into four open reading frames. Despite hepatitis B virus is a DNA virus, it has a high mutation rate due to its replicative strategy, that leads to the production of many non-identical variants at each cycle of replication. In fact, it contains a polymerase without the proofreading activity, and uses an RNA intermediate (pgRNA) during its replication, so error frequencies are comparable to those seen in retroviruses and other RNA viruses rather than in more stable DNA viruses. Due to the low fidelity of the polymerase, the high replication rate and the overlapping reading frames, mutations occur throughout the genome and they have been identified both in the structural and not structural gene. The arise of mutations being to develop of a whole of viral variants called "quasi-species" and the prevalent population, which favors virus replication, was selected by viral fitness, host's immune pressure and external pressure, *i.e.*, vaccination or antiviral therapy. Naturally occurring mutations were found both in acute and chronic subjects. In the present review we examine and discuss the most recent available data about HBV genetic variability and its significance.

Key words: Hepatitis B virus; Mutations; Open reading frames; Molecular biology tools; Liver disease

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Core tip: Hepatitis B virus (HBV) is a global health problem, with almost 2 billion infected persons, many

of whom deemed to develop chronic carrier state and eventually die from cirrhosis or liver cancer. Unlike in other DNA viruses, its high mutation rate and replicative capability arise considerable genetic variability, recently analyzed by molecular biology tools. HBV mutations occur in all four overlapping open reading frames encoding viral polymerase, surface antigen, core and X protein. Understanding the correlation between mutations and liver disease progression is crucial for an effective clinical management in HBV patients with resistance to antiviral drugs, hepatitis B surface antigen escape mutant, "occult" hepatitis and hepatocellular carcinoma.

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INTRODUCTION

Hepatitis B virus (HBV) mutations have been found both in acute and chronic patients and in all the four HBV open reading frames (ORFs - preS/S, polymerase, preCore/core, and X).

The preS/S ORF codes for three different surface molecules that form the surface antigen (HBsAg). This is the main antigen recognized by the immune system, responsible for the attachment of the virus to hepatocytes and the epitope binding the neutralizing antibodies. Point mutations, deletions and also genetic recombinations have been found within the preS/S ORF, which is recognized as the part of HBV genome with the highest heterogeneity. Genetic changes in this region are driven by viral fitness and polymerase infidelity, but also, due to the strict relationships of the products of these genes with the immune system, by host's immune pressure[1,2].

The pol ORF codes for the reverse transcriptase (RT) domain of HBV polymerase that represents the target of the new antiviral agents belonging to the nucleoside/nucleotide analogues and to the acyclic nucleotide analogs (NAs) classes. Under the NAs selective pressure, mutations, collected during the replicative cycles, are selected and confer resistance to NAs. In addition to the high mutation rate, due to the HBV replicative strategy, other factors (viral fitness, potency and genetic barrier of the drugs) are associated to the development of resistance. NAs with high potency and high genetic barrier could prevent resistance generation and should be preferred in HBV therapy. Moreover, due to the overlapping S reading frame, mutations arising in the RT domain cause the appearance of mutations in the preS/S ORF (escape mutants)[1,2].

PreCore/core ORF codes for the core nucleocapsid (HBcAg) and the e antigen (HBeAg) synthesis. Mutations in these sites mainly cause the well-known HBeAg negative hepatitis. The A1762T and the G1764A, responsible for the decreased preCore (PC) mRNA synthesis, were detected in the specific basal core promoter (BCP) and described in patients with HBeAg negative hepatitis. The G1896A mutation caused by a G to A switch is the most prevalent and produces a translation stop codon at amino acid position 28 in the HBeAg sequence, with inhibition of HBeAg synthesis. Moreover, both BCP and stop codons are often associated and recent reports suggest their association with a more severe outcome of hepatitis[1,2].

X ORF encodes for a multifunctional nonstructural protein, originally defined X protein because its functions were unknown and are still unclear. It has been proposed a function in the establishment of infection and viral replication. Furthermore, a role of gene X in the HBV carcinogenesis has been recently hypothesized[1,2].

In the present review we examine and discuss the most recent available data about HBV genetic variability and its significance.

EPIDEMIOLOGY

The virus is transmitted by contact with blood or other body fluids from an infected person. Hepatitis B virus is endemic worldwide and hyper-endemic in many parts of the world.

The prevalence of HBV carriers varies from 0.1% to 2% in low prevalence areas (United States and Canada, Western Europe, Australia and New Zealand), from 3% to 5% in intermediate prevalence areas (Mediterranean countries, Japan, Central Asia, Middle East, and Latin and South America), from 10% to 20% in high prevalence areas (Southeast Asia, China, sub-Saharan Africa). A systematic review focusing on data in the United States estimated that there are 2.2 million individuals with chronic HBV, two-thirds of whom were foreign born[3].

The wide range in HBV carrier rate in different parts of the world is largely related to differences in the age of infection, which is inversely related to the risk of chronicity. The rate of progression from acute to chronic HBV infection is approximately 90% for perinatally acquired infection, from 20% to 50% for infections acquired at 1 to 5 years of age and less than 5% for adult acquired infection[4].

With regard to Europe in 2012, 17329 cases of hepatitis B were reported in 29 countries (no data from Belgium and Liechtenstein), resulting in an overall crude rate of 3.5 per 100000 inhabitants. Of these cases, 2798 (16.1%) were classified as acute infection, 12306 (71.0%) as chronic infection and 1865 (10.8%) as unknown. Three hundred and sixty cases (2.1%) could not be classified in any of these groups. However, due to the differences in surveillance

systems across Europe, these figures are known to be an underestimation of the true situation[5,6].

Ten genotypes have been identified (A-J) on the base of a sequence difference greater than 8% in the entire HBV genome or 4% in the S region. Each genotype is further divided into sub-genotypes when differences in nucleotide sequences are major than 4% but minor than 8%. Interestingly, both genotypes and sub-genotypes are related to clinical course, geographical distribution and mode of transmission. Hyper-endemic areas and at high incidence of hepatocellular carcinoma (HCC) were found in East Asia (genotypes B and C) and in sub-Saharan Africa (genotype A). Moreover, genotypes A and D are prevalent in countries where horizontal transmission is common, *i.e.*, sub-Saharan Africa, Mediterranean, Middle East and India, whilst genotypes B and C are prevalent in countries where vertical transmission is common, *i.e.,* East Asia[7-9].

MORPHOLOGY AND VIRAL GENOME

HBV is a partially double-stranded circular DNA virus that belongs to the *Hepadnaviridae* family. The virus consists of the HBcAg, which contains circular DNA molecule approximately of 3.2 kb, and an outer envelope containing the HBsAg. One of the two strands is incomplete and associated with a DNA polymerase able to complete the strand. This virus is unique among human viral pathogens, since it is a DNA virus that replicates by reverse transcription of an RNA intermediate. The longer strand of HBV DNA (L strand) is a complete circle, whereas the complementary strand is shorter (minus strand). Minus strand DNA is the template for the synthesis of the viral mRNA transcripts. HBV DNA has a very compact coding organization with four partially overlapping ORFs that are translated into seven known proteins: polymerase protein (Pol gene); HBcAg and HBeAg (both from the C gene); large, medium, and small HBsAg (S gene); and the X regulatory protein (X gene). The overlap in the ORFs does not seem to limit variability since all HBV genes have variants. Noncoding regions are not present[10-12].

The first step in the HBV life cycle is its attachment to the hepatocyte through the interaction of its envelope proteins (pre-S1 region) with the host cell receptors.

Then it penetrates in the hepatocyte, uncoating, and the viral genome, organized as relaxed circular partially double stranded DNA (rc DNA), is sent to the nucleus and converted into covalently closed circular DNA (ccc DNA). The cccDNA acts as template for transcription of four co-terminal mRNAs: 3.5 kb pre-core (pre-C) and progenomic RNA (pgRNA), 2.4 kb large surface mRNA, 2.1 kb middle and small surface mRNA and 0,7 kb X mRNA. pgRNA serves as template for the reverse transcriptase and, after being transported to the cytoplasm, encodes viral capside protein and viral polymerase, thus playing an important role in viral genome amplification and replication[1,2].

The latter is transcripted into viral RNA gene products: HBV surface protein, structural core protein, non-structural core protein (secreted HBeAg), X protein and viral polymerase.

After this step the viral assembly occurs (encapsidation by the core protein to form the viral nucleocapsid), followed by the virion secretion or the recycle of the newly generated nucleocapsid into the nucleus for conversion to cccDNA.

The permanence of cccDNA into the hepatocyte nucleus is a basic factor for viral persistence, because it allows for viral replication to restart, either during the antiviral therapy (resistance) or after the antiviral therapy is stopped (reactivation)[13,14].

HBV S-GENE MUTANTS

The pre-S1/S2/S ORFs encode three envelope proteins (large, middle and small) which are determinant for virus assembly and virus attachment to hepatocytes. L protein (pre-S1 domain) is the substrate for viral receptor attachment; M protein (pre-S2 domain) function is not well understood and, finally, S protein (S domain) is commonly referred to as the HBsAg or Australian antigen. The small, the middle and the large proteins are detected as HBsAg. HBsAg protein contains the major B cell epitope, the "a" determinant (121-149 aa)[1].

HBsAg is the surface antigen that is targeted by the antibodies present in vaccinated people and by the antibodies binding to HBsAg in serological immunoassays. It is the major envelop protein, formed by 226 amino acids, it is highly heterogenic, but within the protein there are conserved areas defining the genotype.

The amino acid positions between 99 and 169 are called the major hydrophilic region (MHR), in which the "a" determinant is located (comprising two loops of amino acids, 124-147), that is the main target of neutralizing B cell responses[15,16].

Mutations causing a conformational change within the "a" determinant could affect the antigenicity of HBsAg, essential for inducing protective antibody, and be responsible for escaping vaccine induced immunity, escaping anti HBV immunoglobulin therapy and providing false negative results in serological tests[17-19].

In 1988 HBV S-gene mutants were observed in Italian vaccinated children's sera with the presence of both HBs antigen and anti-HBs antibodies. These children acquired infection from the mother and their S-gene sequences revealed glycine (G) to arginine (A) substitution at position 145, within the *a*-determinant of S-gene, causing conformational changes that allowed for the virus to escape the vaccine-induced response[20]. G145R is the major vaccine-induced immune escape mutation and in the last years an increase of G145R detection has been reported by

several studies, mainly in countries with high rate of endemicity and universal immunization program. Nevertheless, it has been recently demonstrated that the risk of acquiring HBV infection is extremely low in a vaccinated subject. Other mutations were later found in "a" determinant T116N, P120S/E, I/T126A/N/I/S, Q129H/R, M133L, K141EP142S, D144A/E and considered as "immune escape" as well[15].

Similar mutations were also detected in immune-compromised patients and were considered responsible for HBV reactivation by immune escape in previously anti-HBs immune persons. These mutations in the HBsAg can result crucial in failing virus detection in the routine screening.

In recent studies it has been observed that during immunosuppression, some patients, with resolved infection, showed HBV reactivation that in some cases could lead to severe acute hepatitis, synthetic dysfunction, fulminant liver failure and death. In a very recent report, Salpini *et al*[21] showed, that 75.9% of HBV reactivated patients were carriers of more than one HBsAg mutations. 8/13 mutations were located in the major hydrophilic loop (M103I-L109I-T118K-P120A-Y134H-S143L-D144E-S171F) and 5/13 in T cell epitopes belonging to class Ⅰ (C48G-V96A-L175S-G185E-V190A).

In recent years, occult HBV infection (OBI) has been widely investigated. OBI is identified as the persistence of HBV-DNA in HBsAg negative patient's liver with or without other serological markers of previous HBV infection. To explain this phenomenon three mechanisms have been proposed. For two of these, the common factor is the change in the steric configuration in HBsAg molecule, determined by mutations located within the "a" determinant. These modified HBsAg molecules, most commonly, either cannot be detected by commercial available assays or are actually very weakly exposed in the surface of hepatocytes due to a poor recognition by the immune system. Finally, several authors suggest that host immune surveillance and epigenetic mechanisms are probably involved[22].

Some studies report that several and different mutations are correlated with OBI depending on subtypes and also sub-subtypes, or even that OBI associated mutations are unique for each subtype[23,24]. Cassini *et al*[25] suggest that a change in the C695T nucleotide leads to a stop codon in the 181 amino-acid that could be responsible for the strongly reduction of HBsAg production.

Finally, also deletions in the S-region seem to be involved in OBI development, in fact, they can influence the expression, synthesis and secretion of HBsAg[26,27].

According to some authors, mutations in this region might contribute to hepato-carcinogenesis. Lee *et al*[28] discovered the W4P/R pre-S1 mutations. They may be associated with disease severity in male patients chronically infected with HBV genotype C. These W4P/R mutants were significantly related to severe liver diseases [HCC and liver cirrhosis (LC) (12.4%, 19/153 patients) *vs* chronic hepatitis and carrier (1.1%, 1/94 patients), $P < 0.001$]. Interestingly, all the W4P/R mutants were found only in the male gender, not in the female gender, which may in part provide a likely explanation for the relatively high male to female ratio in the incidence of HCC generation in Korean HBV chronic patients.

Other mutations, that usually occur in Pre-S/S region, seem to play an important role in inactivation of the preS2/S region promoter, resulting in interference with HBsAg secretion. As in this region there is the hepatocyte binding site they are associated with occult HBV status as well[29]. Several studies dispute about the important role of pre-S deletions on the progression of liver disease. Above all, it seems that a set of deletions or mutations in different genes is associated with the progression of liver disease. The regions involved are: pre-S, BCP and PC; moreover, it seems that the PC mutations precede the appearance of the others. Pre-S deletions, observed both in pre-S1 and pre-S2 regions, cause a decrease in the synthesis and secretion of small surface antigen which tends to accumulate in the hepatocytes and especially in the endoplasmic reticulum (ER). This supposedly causes an ER stress which in turn causes an oxidative DNA damage that induces mutagenesis and finally HCC. Several other hypotheses have been formulated[30,31]. Wang *et al*[32] suggest that the conspicuous increase of the cyclin A, implicated in the DNA synthesis and centrosome duplication, observed in the HCC tissues and mainly in patients with the pre-S2 deletions, could be activated by the ER stress and could be responsible of the development of HCC. Finally, a study of Yang *et al*[33] demonstrated that, in chronic HBV patients, the pre-S mutants, besides causing ER stress and DNA damage, also cause a vascular endothelial growth factor-A (VEGF-A) overexpression on the ground glass hepatocytes (GGHs). This could be implicated in the preneoplastic GGHs progression to HCC through the activation of Akt/mTOR (mammalian target of rapamycin).

POL-GENE MUTANTS

The goal of treatment in patients with chronic hepatitis B (CHB) is to eliminate the virus, thus reducing the risk of progressive liver damage that leads to the development of complications such as cirrhosis and HCC. However, due to the persistence of cccDNA forms in the infected hepatocytes nucleus, a complete and definitive virus eradication is not achievable.

The currently available drugs, approved for treatment of CHB in many parts of the world, are 2 immuno-modulators (inteferon α-2a and peginteferon α-2a) and 5 antiviral agents belonging to the NAs: lamivudine (LAM-3TC), telbivudine (LdT), entecavir (ETV) and the acyclic nucleotide analogues adefovir

Table 1 Cross-resistance data for the most frequent resistant hepatitis B virus variant

HBV variants	Level of susceptibility				
	Lamivudine	Telbivudine	Entecavir	Adefovir	Tenofovir
Wild-tipe	S	S	S	S	S
M204V	R	S	I	I	S
M204I	R	R	I	I	S
L180M + M204V	R	R	I	I	S
A181A/T	I	S	S	R	S
N236T	S	S	S	R	I
L180M + M204V/I ± I196T ± V173L ± M250V	R	R	R	S	S
L180M + M204V/I ± T184G ± S202I/G	R	R	R	S	S

The amino-acid substitution profiles are shown in the left column and the level or susceptibility is given for each drugs: S (sensitive), I (intermediate/reduced susceptibility), R (resistant). EASL Clinical Practice Guidelines 2012.

dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF)[34]. These last five drugs are inhibitors of RT domain of HBV polymerase.

The viral polymerase/RT is encoded by the largest ORF. This protein arises from the translation product of the 3.5 kb pre-core mRNA and pgRNA, that serves as template for reverse transcriptional synthesis of viral DNA.

Due to the absence of proofreading activity, the HBV polymerases/RT, as already mentioned, leads to the introduction of random mutations into HBV genome. The error rate of HBV polymerase is approximately 1×10^5 to 10^7 base syntheses, as result of the highly error-prone nature of the HBV RT[34,35].

Under the selective pressure by means of the administration of antiviral agents, quasi species of HBV converge on a dominant HBV mutant that can escape selection pressure, creating a drug-resistant HBV strain.

Earlier researches have suggested that LAM is the major cause of YMDD (tyrosine-methionine-aspartate-aspartate) mutations (M204I/V) in the catalytic sites (C domain) within HBV P-ORF[36].

The mutations rtM204I/V (domain C), rtL180M (domain B) and rtA181T/V (domain B) confer resistance to LAM and LdT (Table 1).

M204I/V are often associated with compensatory mutations in other domains such as rtL80V/I, 58 rtI169T,59 rtV173L, rtL180M, rtT184S/G, rtS202I, and rtQ215S which increase viral replication[36-38]. Other proposed compensatory mutations are rtV84M, rt214, rtL217P, rtL229M, rtI233V and rtN238H[37]. Among them, the rtL217P substitution, known to confer replicative advantage if emerging in a wild-type virus, in the context of LAM resistance likely represents a compensatory mutation to boost replication[39] (Table 1).

In fact, compensatory mutations emerge because the selection of resistance-associated changes in the viral polymerase is usually associated with some cost in replication fitness for the virus; these compensatory mutations are important in the setting of antiviral resistance because they "fix" the discriminatory primary drug-resistant mutations into the genetic archive of viral cccDNA, thus providing a "quasi species memory"[38].

ADV resistance is associated with two primary resistant mutations (belonging to the pathway for alkyl phosphonates) in the B and D domain, the rtA181T and the rtN236T. Furthermore, rtI233V is another mutation that has been identified in ADF-resistant HBV variants; its true significance remains contradictory since some authors have confirmed and some have denied its capability to confer resistance[40,41] (Table 1).

Mutations in the B domain of RT, the rtA181T/V, were shown to confer resistance to LAM, LdT and ADV. The rtA181T mutation also encodes a stop codon in the overlapping S reading frame (sW172*) thus resulting in the truncation of the HBsAg proteins. As Warner and Locarnini emphasized, the rtA181T/sW172* variant has a secretory defect and exerts a dominant negative effect on the wild-type HBV virion secretion. This mutation is often present in patients with primary HCC[42-44].

Due to high genetic barrier, ETV and TDF are considered the most potent antiviral agents and at low risk of developing resistance. Indeed, they result to have a mutation incidence rate of 1.2% and 0%, respectively[45]. Long-term monitoring shows HBV resistance to ETV in nucleoside-naive patients is rare through 5 years of therapy. Multiple mutations are required to obtain high-level resistance to ETV. Those usually involved in ETV resistance are rtL180M + M204V and another among rtI169T, rt184G/S, rtS202I/G and rtM250V; actually, ETV resistance appears in LAM treated patients in which the rtL180M and M204V mutations were formerly present[38] (Table 1).

To date, there have been no confirmed reports of resistance selection during treatment of CHB with TDF in mono-infected individuals. Kitrinos *et al*[46] in their study report that TDF mono-therapy maintained effective viral suppression over up to 6 years of continuous therapy without selecting TDF resistance. Recently, a complex TDF-resistance associated mutation pattern, including the rtR192PR substitution, very close to the site of the rtA194T mutation which has been found to confer TDF resistance *in vitro,* has been reported in a HIV-HBV co-infected individual failing TDF[47].

Table 2 Impact of drug resistant mutations in the the hepatitis B virus Pol on the hepatitis B surface antigen

Antiviral drugs	Resistance mutations	HBsAg corresponding changes
Lamivudine (LAM[2])	rtL180M	No change
Tebivudine (LdT[2])	rtM204V	sI195M
	rtM204I[2]	sW196[1]/S/L
Adefovir (AdV)	rtA181T[2]	sW172[1]
Tenofovir (TDF)	rtA181T[2]	sW172L
LAM[2]	rtA181V[2]	sL173F
	rtN236T	After end of HBs open reading frame
Entecavir (ETV)	rtI169T	sF161H/L
	rtT184A	No change
	rtT184C	sL175F + sL176V
	rtT184I	No change
	rtT184G	sL176V
	rtT184S	sL175F
	rtT184M	sL176[1]
	rt184L	sL175F
	rtS202C	No change/sS193F
	rtS202I	sV194F/S
	rtS202G	No change/sS193L
	rtM250I	After end of HBs open reading frame
	rtM250V	After end of HBs open reading frame

[1]Stop codon; [2]Cross-resistance. HBsAg: Hepatitis B surface antigen. Modified from Zoulim *et al*[38].

The common mutations that confer resistance to LAM and LdT (*e.g*., rtM204V/I, rtL180M) give cross-resistance to other L-nucleosides and reduce sensitivity to ETV but not to ADV or TDF. Conversely, mutations causing resistance to ADV (rtA181T/V, rtN236T) and TDF generally do not give rise to resistance to L-nucleosides and ETV. Both the L-nucleosides (LMV and LdT) and the alkyl phosphonates (ADV and TDF) also select the mutation rtA181T/V, thereby making it a marker for multidrug resistance[36-38] (Table 1).

Further research has revealed that strains with YMDD mutations also exist in patients with chronic HBV infection not previously treated with lamivudine[48,49].

A recent research showed that spontaneous YMDD mutations were detected in LC and HCC patients. Moreover, it has been demonstrated that in genotype C, HCC patients had a significantly higher spontaneous YMDD mutation rate than LC patients, and that genotype C was associated with a higher risk for the development of HBV-related HCC than patients infected by other HBV genotype (P = 0.013, 95%CI: 1.540-39.264). This may have been caused by different genotype strains having different biological properties, pathogenicity and carcinogenicity[50].

Furthermore, the rate of viral breakthrough tended to be lower in patients without natural YMDD mutations than in those with natural YMDD mutations. Naturally occurring YMDD mutations are found in a large proportion of CHB patients who have not undergone anti-viral therapy. The incidence of YMDD mutations may be correlated with the HBeAg status and the HBV DNA level. These results also suggest that LAM therapy improved the clinical course in HBV patients with natural YMDD mutations[51].

The HBV polymerase (Pol) gene overlaps the HBsAg in a frame-shifted manner with the result that drug resistant mutations in the HBV Pol can directly impact on the HBsAg and its function. Therefore, drug resistance mutations in the polymerase gene may result in the production of mutations and stop codons in the envelope gene leading to modified viral secretion, infectivity and creating both viral escape to anti-HBs antibodies[38] and modified HBsAg molecule not detected by screening tests (Table 2). About this last topic, the study of Hsu *et al*[52] found that the P120A mutation in the HBsAg gene, selected during LAM therapy in 6/11 samples patient, was responsible for HBsAg detection failure, misinterpreted as HBsAg clearance.

Through a molecular analysis performed in HIV-HBV co-infected and HBsAg-negative patients, Amini-Bavil-Olyaee in 2009 revealed an unusual HBV polymerase mutation (rtV191I), during TDF therapy, conferring simultaneously immune escape by HBsAg negativity and resistance to LAM, but not TDF. Due to the overlapping surface antigen the rtV191I mutation also created a stop-codon in sW182s, deleting the last 44 amino acids of the HBsAg, which resulted HBsAg negative in diagnostic serum assays[53].

Interestingly, neither the ADV-associated resistance mutation rtN236T nor the TDF-associated resistance mutation rtA194T, selected only *in vitro*, cause changes in the HBV surface gene[54].

HBV mutants carrying drug and vaccine resistance may represent a considerable individual risk and public health concern.

With regard to the best treatment strategy after HBV resistance, the international practice guidelines recommend the use of a nucleoside/tide analogue with high antiviral potency and high genetic barrier, such as ETV or TDF. Nevertheless, incomplete response to ETV therapy has been reported[55].

PRE CORE/CORE MUTANTS

Pre-Core/Core region encodes for two proteins, one structural, the HBcAg, that forms the nucleocapsid, and the HBeAg that is a secretion protein[2-56].

HBeAg is the marker of HBV replication and infectivity. In the natural course of HBV chronic infection, the loss of HBeAg expression and the appearance of antibodies directed against it (anti-HBe) usually represent the end of viral replication and the resolution of hepatitis. Mutations in the pre-core and core regions cause HBeAg-negative chronic hepatitis B with presence of anti-HBe, in which replicative infection continues and HBV-DNA remains detectable (> 2000 IU/mL)[2,15,56].

HBeAg negativity is due to basal core promoter

(BCP) and precore (PC) mutations that respectively modulate HBeAg secretion during transcription and stop HBeAg production[2,15,56].

Recently, in Korea, Lee *et al*[57] described 36 prospectively enrolled patients with acute hepatitis B, 20 of which, infected with HBV genotype C, showed detectable HBV DNA. Among them, 4 patients had BCP mutations, and two had PC mutations. Platelet counts were significantly lower in the 4 patients with PC/BCP mutations compared to those with wild type. The A1762T and the G1764A, responsible for the decreased PC mRNA synthesis, were the typical specific BCP mutations detected and described, mainly together, in patients with HBeAg negative hepatitis. These two mutations were first found in a study of Baptista *et al*[58] aimed at investigating the presence of mutations responsible for the HBeAg negativity and their possible role in hepato-carcinogenesis in the HBeAg negative patients. This study showed that these two mutations produced a decrease in the HBeAg secretion and had a significant role in hepato-carcinogenesis[59]. The increased risk of HCC in patients harboring a virus with the A1762T and the G1764A was confirmed by several studies but the mechanism of oncogenesis remains unknown[59-63]. Furthermore, Yang *et al*[64] investigated the risk of HCC considering, in addition to BCP mutations, also HBV genotypes and PC mutations. They proved that the highest risk of HCC development depends on genotype (mostly genotype C), and on the presence of the A1762T and G1764A BCP mutations and of the G1896A PC mutation.

In addition to the A1762T and G1764A mutations, other BCP mutations have been identified: the T1753C, and the C1766T. Basically, these mutations reduce the HBeAg synthesis and enhance viral replications in liver cells, often in association with more severe and advanced liver disease[65].

Some of these mutations (T1753C, A1762T and 1764A), together with A1752G, A1846T, G1896A and G1899A, were significantly correlated with HBeAg seroconversion; in a recent work the authors showed significant differences between HBeAg positive and HBeAg negative child patients groups. But the frequencies of the mutations in HBeAg-negative child patients were significant lower than in HBeAg negative adult patients, because the role of BCP/PC mutations is less important in the early phases of HBeAg seroconversion[66].

The main prevalent PC region mutations collected over the years in various works are the G1896A and the G1899A, alone or associated. The G1896A mutation is due to a G to A switch that produce a translation stop codon at amino acid position 28 in the HBeAg sequence, with inhibition of its synthesis. This mutation was often found in non-A genotypes associated with the mutation C1858T, whose onset is eased by typical viral structure of certain genotypes (B, D, E, C, F). Also these mutations have been first found in Mediterranean Countries, where the majority of patients are genotype D carriers.

In a longitudinal study on 99 HBV- DNA positive patients, all genotype D, HBeAg negative and with PC G1896A mutation, Besharat observed that they still had a detectable HBV-DNA even after 7 years of monitoring, unlike the patients with the wild type PC sequence[67].

X-GENE MUTANTS

Gene X encodes for a multifunctional nonstructural protein, originally defined X protein because its function was unknown and even now are unclear. It has been proposed a function in the establishment of infection and viral replication. Furthermore, a role of gene X in the HBV carcinogenesis has been recently hypothesized[68].

The HBX gene overlaps with the core promoter region and mutations here in this gene may alter the functions of the HBX protein, playing an important role in HBV replication and hepato-carcinogenesis. According to Yan *et al*[69], the HBX mutants linked with core promoter mutations may regulate p53 through a S-phase kinase associated protein 2 (SKp2), promoting or preventing cellular transformation and proliferation.

In HBX region, twelve mutations were associated with hepato-carcinogenesis, suppression of HBeAg secretion and increase of viral DNA synthesis[70].

CONCLUSION

In the last decade, mainly due to molecular biology studies, a lot of information about HBV life cycle, genetic variability and pathogenesis has been achieved. HBV genomic sequencing, back in 1988, allowed Zanetti *et al*[20] to discover the G145R mutation within the "a" determinant of S gene, the first escape mutant identified. Other escape mutants have been detected afterwards and the relevant role of other mutations has been established in immune compromised patients and in OBI infection. Sequencing of *pol* gene, especially performed to drive clinicians to the better treatment, not only has allowed to achieve knowledge on the mutations able to confer resistance to the new NAs, some of which are often related with hepato-carcinogenesis, but, considering the overlapping of the *pol* gene with the S gene, also to discover other escape mutants or stop codons in this site. Sequencing also allowed to identify mutations responsible of HBeAg-negative chronic hepatitis B and finally to identify mutations, deletions and insertion in X gene probably associated with hepato-carcinogenesis, suppression of HBeAg secretion and increase of viral DNA synthesis. Nevertheless, further studies are needed in the field of HBV genetic variability, especially to investigate on the role of X gene, about which there are still too few data which also need to be confirmed. Finally,

further studies are also needed to understand whether and how much genotypes and sub-genotypes could influence the response to treatment, the appearance of viral variants and the risk of cirrhosis and HCC. It is possible to hypothesize that additional knowledge above viral variants, genotypes and sub-genotypes could be considered into clinical decision.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Association between hepatitis B and metabolic syndrome: Current state of the art

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Abstract

Chronic hepatitis B (CHB) is a global health issue that increases the risk of liver cirrhosis and hepatocellular carcinoma in infected patients. Metabolic syndrome (MetS) is a disease endemic mostly to the developed countries. It is associated with high cardiovascular mortality and morbidity, diabetes mellitus as well as cancer. In this manuscript, we systematically review the published data on the relationship between MetS and CHB infection. Multiple studies have described highly variable correlations between CHB on one hand and MetS, non-alcoholic fatty liver disease and dyslipidemia on the other. No association between CHB and diabetes mellitus or atherosclerosis has been described as of now. The presence of MetS in patients infected with hepatitis B virus increases the risk of fibrosis, cirrhosis and hepatocellular carcinoma. Appropriate lifestyle, but also pharmacological interventions are needed to prevent the development of these complications.

Key words: Hepatitis B; Nonalcoholic fatty liver disease; Fibrosis; Cirrhosis; Metabolic syndrome; Hepatocellular carcinoma

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Core tip: Currently, no clear relationship between chronic hepatitis B (CHB) and the prevalence of metabolic syndrome (MetS) could be established, but observations on large patient cohorts reveal some interesting patterns. Surprisingly, male patients with CHB may have lower prevalence of MetS than patients without CHB, but this has not been observed in females. Furthermore, CHB is probably not associated with higher risk of type 2 diabetes mellitus or atherosclerosis. Regarding the clinical outcomes, available data do not sufficiently reveal all of the possible interactions between MetS, its individual components and CHB.

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INTRODUCTION

Approximately 2 billion people are infected with hepatitis B virus (HBV) during their lifetime. Around 350-400 million people are infected at any given moment. One hundred twenty-five million of these come from China. Acute hepatitis B infection progresses in a proportion of patients into chronicity. Chronic hepatitis B (CHB) subsequently increases the risk of liver cirrhosis and hepatocellular carcinoma (HCC). In CHB patients HCC could occur even without the presence of cirrhosis[1]. More than one million patients with CHB die due to liver failure or HCC annually[2].

Metabolic syndrome (MetS) has various definitions, however all these definitions stress the presence of abdominal obesity in conjunction with other parameters. Widely used criteria from the International Diabetes Federation define central obesity as waist circumference ⩾ 94 cm for males and ⩾ 80 cm for females in western population; ⩾ 90 cm for males ⩾ 80 cm for females for Asian population excluding Japanese, or BMI > 30 kg/m^2). Two or more of the following criteria also need to be fulfilled: (1) "Raised triglycerides ⩾ 150 mg/dL (1.7 mmol/L) or specific treatment for this lipid abnormality; (2) reduced HDL cholesterol < 40 mg/dL (1.03 mmol/L) in males < 50 mg/dL (1.29 mmol/L) in females or specific treatment for this lipid abnormality; (3) raised blood pressure systolic ⩾ 130 or diastolic ⩾ 85 mmHg or treatment of previously diagnosed hypertension; and (4) raised fasting plasma glucose ⩾ 100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes If above 5.6 mmol/L or 100 mg/dL, oral glucose tolerance test is strongly recommended but is not necessary[3]."

Patients suffering from MetS have higher risks of cardiovascular morbidity and mortality, diabetes mellitus and cancer[4,5]. Nonalcoholic fatty liver disease (NAFLD) is currently considered a hepatic manifestation of MetS.

ASSOCIATION BETWEEN THE PREVALENCE OF MetS AND CHB

The presence of MetS in patients with CHB (HBsAg positive) was evaluated in 10 published studies[6-15]. The results are summarized in Table 1. The majority were done in Asia, one study analyzed a large population database in the United States and two papers report the data from central Europe. The data presented in these studies are very heterogeneous. In three out of seven Asian studies, authors reported an inverse correlation between the prevalence of MetS and CHB in the whole cohort[6-8]. In the next two Asian studies, this inverse correlation between CHB and MetS was present, after adjustment, only in males, while no correlation was observed in females[10,15]. One study did not found any association between the prevalence of these two diseases[9], and in the retrospective cohort from Shanghai authors found that patients with CHB had a higher prevalence of MetS compared to the uninfected controls[12]. The study from United States that evaluated the proposed relationship in the large population database (NHANES Ⅲ) found previously documented inverse correlation in the whole cohort and males, however this correlation was not present in women. The results were adjusted for race and other confounders, however it is prudent to mention that roughly 80% of controls were non-Hispanic whites, but the prevalence of hepatitis B was higher in other races[11]. Although not mentioned specifically in this study, it is known that a significant proportion of CHB patients in the United States is of Asian descent[16]. No relationship between CHB and MetS was found in both studies from Europe, however, the study by Jarčuška *et al*[13] and Janicko *et al*[14] included only very specific young Roma population, but the second study expanded this population with also majority Caucasian population. A meta-analysis of four, previously mentioned studies from Asia[6,8-10] was performed by Wang *et al*[17]. Altogether, authors included 10015 patients with CHB and 79475 controls. Despite the findings of the original studies, no difference in the prevalence of MetS in the CHB patients and controls was found (OR = 0.82; 95%CI: 0.66-1.02 by random effect model; heterogeneity: I-squared 84.8%)[17].

As is evident from the conflicting results of published studies, currently no clear relationship between CHB and MetS prevalence could be established. However, it is necessary to note that male population with CHB may have lower prevalence of MetS and the prevalence of MetS increases with age not only in uninfected patients but also in patients with CHB[13].

The presence of antibodies against HBV core antigen (antiHBc) without HBsAg positivity signifies previous contact with hepatitis B. Four studies examined the relationship between antiHBc positivity and the presence of MetS. In the NHANES Ⅲ cohort no difference in the MetS prevalence between antiHBc positive patients and controls was found (OR = 0.87, 95%CI: 0.69-1.08, adjusted for age, sex, race, smoking and alcohol status)[11]. Another cross-sectional study from Taiwan included 8226 subjects with mean age 19.2 ± 2.3 years. AntiHBc positive patients in this study had 58% higher risk of having MetS (P < 0.05)[18]. Similarly, antiHBc positive subjects had higher prevalence of MetS compared to antiHBc negative controls (29.8% *vs* 22%, P = 0.008) also in the study from central Europe. However, antiHBc positive patients were also significantly older compared to antiHBc negative patients[13]. In the specific population of young Roma

Table 1 Association between chronic hepatitis B virus infection and metabolic syndrome

Ref. (Region)	Race	Study design	HBV patients/ controls	Prevalence MS in HBV patients/controls	Result/statistical significance
Jan *et al*[6] (Taiwan)	Asian	Population-based Cross-sectional study	5995 HBV patients/ 47533 controls	8.0%/10.9%	Inverse correlation between MS and HBV infection OR = 0.72 (0.65-0.79); $P < 0.001$ aOR = 0.84 (0.76-0.93); $P < 0.001$[1]
Luo *et al*[7] (China)	Asian	Cross-sectional study	858 HBV patients/ 6579 controls	5.9%/8.8%	Inverse correlation between MS and HBV infection OR = 0.65 (0.48-0.88); $P = 0.003$
Wong *et al*[8] (Hong Kong, China)	Asian	Case series	91 HBV patients/ 922 controls	11.0%/20.2%	Inverse correlation between MS and HBV infection; $P = 0.034$
Li *et al*[9] (Taiwan)	Asian	Case series	3408 HBV patients/ 22897 controls	13.4%/14.0%	No correlation between MS and HBV infection
Chung *et al*[10] (South Korea)	Asian	Cross-sectional study	521 HBV patients/ 8953 controls	19.5%/20.8% in men 14.3%/13.7% in women	Inverse correlation between MS and HBV infection in men only after adjustation OR = 0.92 (0.72-1.17); $P = 0.492$; NS aOR = 0.75 (0.57-0.98); $P = 0.033$[b] No correlation between MS and HBV infection in women OR = 1.05 (0.56-1.96) NS aOR = 0.80 (0.38-1.66); $P = 0.545$; NS[2]
Jinjuvadia *et al*[11] (United States)	Caucasian (80%)	Large population database	593 594 HBV patients/7280620 patients with past exposure to hepatitis B/138283905 controls	10.4%/25.6% total	Inverse correlation between MS and HBV infection in all patients OR = 0.34 (0.13-0.87) aOR = 0.32 (0.12-0-82); $P = 0.019$[c] Inverse correlation between MS and HBV infection in men OR = 0.13 (0.04-0.44) aOR = 0.14 (0.04-0.55)[3] No correlation between MS and HBV infection in women OR = 0.89 (0.30-2.65) aOR = 0.73 (0.22-2.46)[3]
Zhou *et al*[12] (China)	Asian	Retrospective cohort study	480 HBV patients/ 496 controls	24.5%/10.5%	Correlation between MS and HBV infection OR = 2.46 (1.77-3.41) aOR = 2.27 (1.52-3.38)[4]
Jarčuška *et al*[13] (Slovakia)	Caucasian + Roma	Cross-sectional study	66 HBV patients/ 789 controls	24.6%/24.7%	No correlation between MS and HBV infection; $P = 0.561$; NS
Janicko *et al*[14] (Slovakia)	Roma	Cross-sectional study	55 HBV patients/ 387 controls	27.8%/29.6%	No correlation between MS and HBV infection; $P = 0.785$; NS
Choi *et al*[15] (South Korea)	Asian	Population database	209 HBV patients/ 4899 controls	23.4%/31.5% in men 18.6%/23.7% in women	Inverse correlation between MS and HBV infection in men only after adjustation OR = 0.66 (0.42-1.05); $P = 0.079$; NS OR = 0.61 (0.375-0.998); $P = 0.049$[5] No correlation between MS and HBV infection in women OR = 0.74 (0.44-1.22); $P = 0.235$; NS aOR = 0.70 (0.40-1.21); $P = 0.197$; NS[5]

[1]Adjusted for age and sex; [2]Adjusted for age, body mass index, alaninaminotranferase, alcohol intake, smoking, exercise, family income and educational status; [3]Adjusted for age, sex, race, smoking and alcohol status; [4]Adjusted for age, gender, smoking, passive smoking, alcohol consumption, high-energy food intake, fresh fruit and vegetable intake, physical activity; [5]Adjusted for age, location, smoking habits, alcohol consumption, exercise habits, income status, and education levels. MS: Metabolic syndrome; NS: Not significant; HBV: Hepatitis B virus; OR: Odds ration; aOR: Adjusted odds ratio.

people, no significant difference in the MetS prevalence was found (31.9% *vs* 26.7%, not significant)[14].

Very limited data suggest lower prevalence of MetS in subjects vaccinated against hepatitis B. In the above mentioned study from Taiwan, antiHBs positive, antiHBc negative subjects had lower prevalence of MetS compared to antiHBs negative controls (OR = 0.76, 95%CI: 0.6-0.96, adjusted for age, gender and BMI). Due to the design of the study, it is difficult to determine if this association is only arbitrary or has a clinical foundation[18].

LIPOPROTEIN METABOLISM IN THE PATIENTS WITH CHB

The lipid profile in the serum of patients with CHB has recently drawn significant attention[6-11,13-15,19-22]. An overview of the published studies is in the Table 2. The levels of total cholesterol were significantly lower in most of the CHB patients compared to controls in practically all of the published studies. Two of the studies also reported lower levels of apolipoprotein B100, which is the principal protein component of

Table 2 Levels of lipoproteins in chronic hepatitis B virus patients and controls

Ref.	Laboratory parameter	HBV patients *vs* controls statistical significance	HBV patients *vs* controls
Su *et al*[19]	Total cholesterol	$P < 0.05$	181.7 ± 29.8 mg/dL *vs* 186.8 ± 33.3 mg/dL
	LDL-C	NS	108.7 ± 25.9 mg/dL *vs* 109.4 ± 28.6 mg/dL
	HDL-C	$P < 0.01$	53.4 ± 11.6 mg/dL *vs* 56.5 ± 13.5 mg/dL
	TG	NS	99.2 ± 54.0 mg/dL *vs* 102.7 ± 57.6 mg/dL
Jan *et al*[6]	TG	OR = 0.64 (0.60-0.69)	
	HDL-C	OR = 0.89 (0.80-0.99)	
Luo *et al*[7]	TG	OR = 0.62 (0.53-0.72); $P = 0.002$	
	HDL-C	NS	
Chen *et al*[20]	Cholesterol	$P < 0.001$	
	TG	$P < 0.001$	
Wong *et al*[8]	Total cholesterol	$P = 0.004$	4.9 ± 0.8 mmol/L *vs* 5.2 ± 1.0 mmol/L
	LDL-C	NS	2.9 ± 0.8 mmol/L *vs* 3.0 ± 0.9 mmol/L
	HDL-C	NS	1.5 ± 0.4 mmol/L *vs* 1.5 ± 0.4 mmol/L
	TG	$P = 0.027$	1.0 (0.1-2.9) mmol/L *vs* 1.1 (0.3-21.3) mmol/L
Hsu *et al*[21]	LDL-C	NS	
	HDL-C	aOR = 0.004 (0.001-0.017); $P < 0.001$[1]	
	TG	aOR = 0.107 (0.054-0.213); $P < 0.001$[1]	
Li *et al*[9]	Total cholesterol ⩽ 45 yr in women	$P < 0.001$	178 mg/dL *vs* 174 mg/dL
	Total cholesterol > 45 yr in women	$P = 0.040$	201 mg/dL *vs* 205 mg/dL
	LDL-C ⩽ 45 yr in women	$P = 0.040$	103.5 mg/dL *vs* 101.2 mg/dL
	LDL-C > 45 yr in women	NS	123.6 mg/dL *vs* 126.8 mg/dL
	HDL-C ⩽ 45 yr in women	$P < 0.001$	63.3 mg/dL *vs* 61.5 mg/dL
	HDL-C > 45 yr in women	NS	60.1 mg/dL *vs* 59.4 mg/dL
	TG ⩽ 45 yr in women	NS	67 mg/dL *vs* 67 mg/dL
	TG > 45 yr in women	$P < 0.001$	85 mg/dL *vs* 93 mg/dL
	Total cholesterol ⩽ 45 yr in men	NS	183 mg/dL *vs* 182 mg/dL
	Total cholesterol > 45 yr in men	$P < 0.001$	188 mg/dL *vs* 197 mg/dL
	LDL-C ⩽ 45 yr in men	NS	49.8 mg/dL *vs* 49.7 mg/dL
	LDL-C > 45 yr in men	$P < 0.001$	117.6 mg/dL *vs* 123 mg/dL
	HDL-C ⩽ 45 yr in men	NS	51 mg/dL *vs* 51 mg/dL
	HDL-C > 45 yr in men	NS	49.8 mg/dL *vs* 49.7 mg/dL
	TG ⩽ 45 yr in men	$P = 0.017$	100 mg/dL *vs* 104 mg/dL
	TG > 45 yr in men	$P < 0.001$	102 mg/dL *vs* 116 mg/dL
Liu *et al*[22]	Total cholesterol	$P < 0.05$	193 ± 36 mg/dL *vs* 197 ± 36 mg/dL
	LDL-C	$P < 0.05$	124 ± 31 mg/dL *vs* 126 ± 36 mg/dL
	HDL-C	NS	53 ± 16 mg/dL *vs* 53 ± 16 mg/L
	TG	NS	126 ± 129 mg/dL *vs* 131 ± 87 mg/dL
Chung *et al*[10]	TG in men	$P < 0.001$	4.59 ± 0.48 mg/dL *vs* 4.75 ± 0.52 mg/dL
	HDL-C in men	$P = 0.039$	3.81 ± 0.26 mg/dL *vs* 3.84 ± 0.25 mg/dL
	TG in women	NS	4.45 ± 0.30 mg/dL *vs* 4.50 ± 0.50 mg/dL
	HDL-C in women	NS	4.01 ± 0.20 mg/dL *vs* 3.97 ± 0.24 mg/dL
Jinjuvadia *et al*[11]	TG	NS (total, in men, in women)	
	HDL-C (total)	OR = 0.37 (0.15-0.91)	
	HDL-C in men	NS	
	HDL C in women	OR = 0.26 (0.07-0.93)	
Jarčuška *et al*[13]	Total cholesterol	$P = 0.001$	4.54 ± 0.84 mmol/L *vs* 5.00 ± 0.99 mmol/L
	LDL –C	$P = 0.001$	2.29 ± 0.58 mmol/L *vs* 2.60 ± 0.68 mmol/L
	HDL-C	NS	1.19 ± 0.35 mmol/L *vs* 1.19 ± 0.41mmol/L
	TG	NS	1.11 ± 0.59 mmol/L *vs* 1.31 ± 0.91 mmol/L
	ApoB100	$P = 0.013$	0.71 ± 0.21 g/L *vs* 0.77 ± 0.23 g/L
Janicko *et al*[14]	Total cholesterol	$P = 0.035$	4.45 ± 1.21 mmol/L *vs* 4.71 ± 1.23 mmol/L
	LDL –C	NS	2.20 ± 0.88 mmol/L *vs* 2.50 ± 0.90 mmol/L
	HDL-C	NS	1.10 ± 0.53 mmol/L *vs* 1.10 ± 0.36 mmol/L
	TG	NS	1.02 ± 1.56 mmol/L *vs* 1.15 ± 1.75 mmol/L
	ApoB100	$P = 0.025$	0.66 ± 0.26 g/L *vs* 0.74 ± 0.29 g/L
Choi *et al*[15]	TG in men	OR = 0.63 (0.40-0.99); $P = 0.043$	
	HDL-C in men	NS	
	TG in women	OR = 0.34 (0.17-0.69); $P = 0.003$	
	HDL-C in women	NS	

[1]Multivariate analyses using logistic regression, the status of HBsAg positivity as the dependent variable, age, sex, body mass index, serum TG, LDL-C, HDL-C and alaninamonotransferase level as independent variables were performed. NS: Not significant; HBV: Hepatitis B virus; OR: Odds ration; TG: triglycerides; HDL-C: High density lipoproteins; LDL-C: Lowe density lipoproteins; ApoB100: Apolipoprotein B100; aOR: Adjusted odds ratio.

low and very low-density lipoprotein particles[13,14]. The data on individual lipoprotein classes are more conflicting. Currently published studies mostly did not find any difference in the levels of low-density lipoproteins (LDL) in patients with CHB and controls. Nevertheless, three studies did report significant differences in LDL values[9,13,22], however the direction and magnitude of these differences differed greatly between studies and subgroups within individual studies. Same conclusions can be drawn from the published data about triglycerides and high-density lipoproteins (Table 2).

The risk of atherosclerosis related outcomes has been evaluated in only one large study from Taiwan that included 3931 CHB patients and 18541 controls followed for 17 years. The HBsAg seropositivity did not increase the risk of coronary heart disease, cerebrovascular disease and atherosclerosis in general[23].

No simple reason for these changes in the lipoprotein metabolism in CHB patients has been confirmed in the literature. However, multiple proposed explanations exist. It has been shown that total cholesterol correlates with liver function and prognosis in patients with advanced liver disease. Therefore, at least in a proportion of patients, the low total cholesterol could be associated with incipient liver failure. Hepatitis B infection also interferes with the hepatocyte metabolism. It has been known for some time that HBV modifies the expression of host genes. Particularly the genes for enzymes of lipid biosynthesis pathways were the largest upregulated category in one published murine model[24]. On the other hand, data from hepatoma cell cultures suggest that hepatocytes infected with HBV have lower concentrations of apolipoprotein mRNA[25]. The binding of apolipoprotein H to the HBsAg could also result in the lower plasma apolipoprotein levels[26]. Therefore, despite the lack of strong cytotoxic effect, HBV infection profoundly alters the metabolism of infected hepatocytes.

CHB, INSULIN RESISTANCE AND DIABETES MELLITUS

Chronic hepatitis C is an important risk factor for insulin resistance that accelerates fibrogenesis in the liver[27]. Moreover, patients with hepatitis C and insulin resistance have poorer response to antiviral treatment[28]. This relationship in CHB patients is not so straightforward. The insulin resistance was not associated with HBsAg positive patients in a study by Wang *et al*[29] from Taiwan. However, another study from Korea reported that patients with CHB had higher levels of fasting insulin, HOMA-IR index and lower QUICKI index[30]. In a recent meta-analysis of 15 studies, no increase in the risk of type 2 diabetes mellitus (T2DM) attributable to CHB without cirrhosis was reported. However, increased risk of T2DM was reported in CHB patients with liver cirrhosis compared to CHB patients without cirrhosis (OR = 1.74, 95%CI: 1.43-2.13). Authors of this meta-analysis concluded that HBV itself might not be pro-diabetic[31]. Shen *et al*[32] tried to identify the risk factors for T2DM in patients infected with hepatitis B. Multivariate analysis revealed that besides general risk factors (family history, low education level, elevated triglycerides, gamma-glutamyl transferase, and alcoholic steatosis), three hepatitis B related risk factors (high viral load, long duration of infection and presence of cirrhosis) contributed independently to the risk of T2DM.

CHB AND NAFLD

Insulin resistance is the principal pathophysiological mechanism behind the MetS. Although not officially included in the definition of MetS, NAFLD is very prevalent is patients with MetS and its clinical prevalence is estimated around 20% in the general population of developed countries. Histological prevalence could be even higher. In a series of consecutive liver biopsies from potential liver donors, the prevalence of histological changes associated with NAFLD was 50%[33-35]. Non-alcoholic fatty liver disease could progress to non-alcoholic steatohepatitis (NASH) and liver cirrhosis. The prevalence of NASH is estimated around 2%-3% of general population. It progresses to cirrhosis in 20%-25% of the cases. The mortality of NASH related cirrhosis is estimated around 40% mostly due to liver failure or HCC[36]. Besides liver related mortality, the presence of NASH also increases the risk of total and cardiovascular mortality[37]. The definitive diagnosis of NAFLD is confirmed by quantification of the fat in the liver biopsy specimen, which should be more than 5%[38]. The liver biopsy is not feasible for the routine diagnosis of NAFLD and ultrasound is commonly used instead. The correlation between ultrasound and biopsy is very good (Spearman's coefficient: 0.80; 95%CI: 0.71-0.88, $P < 0.001$)[39]. Another relatively sensitive and specific noninvasive test for NAFLD is proton-magnetic resonance spectroscopy (^{1}H-MRS) that determines intrahepatic triglyceride content[40]. The NAFLD-associated fibrosis could also be evaluated noninvasively by transient elastography, serum biomarkers or the combination of both[41].

In the large study that included 33439 subjects who received health check-up at single center in Taiwan, NAFLD was found in 43.9% in general uninfected population compared to only 38.9% in patients with CHB. This result was also significant in lean and overweight subgroups separately[42]. However, two other, smaller studies did not find significant difference in the prevalence of NAFLD between CHB and uninfected patients[29,43]. Study by Wong *et al*[8] determined the NAFLD prevalence in 91 patients with CHB and 922 controls by proton magnetic spectroscopy. The intrahepatic triglyceride content was significantly lower in infected patients compared

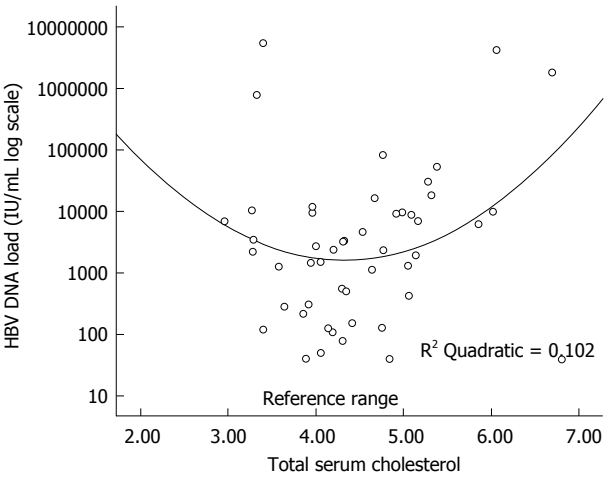

Figure 1 Association between total cholesterol and hepatitis B virus DNA load. Adapted from Janicko *et al*[14] (2014).

to the controls (1.3% *vs* 2.1%, $P < 0.001$). When the presence of NAFLD was determined using 5% cut-off for triglyceride content, patients with CHB had markedly lower prevalence compared to uninfected patients (13.5%; 95%CI: 6.4%-20.6% *vs* 28.3%; 95%CI: 25.3%-31.2%).

Some histological data on the prevalence of NAFLD in CHB patients are available, however bioptic studies naturally did not include the control group. Machado *et al*[44] published a meta-analysis of 17 studies that assessed hepatic steatosis prevalence by histology and compared the results to hepatitis C patients. Overall, NAFLD prevalence was 29.6%, patients with CHB had significantly lower prevalence of hepatic steatosis compared to patients with hepatitis C (OR = 0.55, 95%CI 0.45-0.67, $P < 0.001$) and the prevalence was comparable to uninfected patients. In CHB patients, the risk factors for hepatic steatosis were male sex, higher BMI, diabetes mellitus, higher levels of serum glucose, triglycerides, total cholesterol and higher alcohol consumption. On the other hand, the steatosis was negatively associated with HBV DNA. Other virus related factors, such as HBeAg, genotype or histology did not have any association with hepatic steatosis. In the study by Zheng *et al*[45], that included only CHB patients, hepatic steatosis also correlated well with fasting insulin levels, however only BMI and total cholesterol were predictors of hepatic fibrosis.

The combination of CHB and NAFLD could potentially accelerate the development of fibrosis. This was assessed in the study by Peng *et al*[46] who found that in patients with CHB and hepatic steatosis the stages of fibrosis were independently associated only with histology activity index and HBV DNA. No significant association between hepatic steatosis and the stages of cirrhosis was reported in this study. Above-mentioned meta-analysis confirmed that the presence of NAFLD does not worsen the necroinflammation or the degree of fibrosis in patients with CHB[44].

In conclusion, the prevalence of NAFLD is comparable, but may even be lower in patients with CHB than in general population. Surprisingly, the presence of NAFLD is not associated with the severity of liver fibrosis.

HEPATITIS B VIRAL LOAD AND MetS

Multiple studies on animal models have shown that HBV influences metabolism, most likely by changes in the gene expression. Some authors therefore use the term "metabolovirus", because the regulation of gene expression of HBV and key metabolic genes in hepatocytes is very similar[47]. It is known that HBV DNA load increases the risk of liver cirrhosis and HCC[48,49]. The presence of a relationship between MetS and HBV DNA viral load has not been confirmed to date. Some studies did not find any relationship[50,51], while another proposed an inverse correlation between HBV DNA and liver steatosis[52] that has been shown also in the already mentioned meta-analysis. Pooled data from seven studies in this meta-analysis showed significant negative effect of HBV load on the prevalence of hepatic steatosis[44]. On the other hand, in a study from our group CHB patients with MetS had significantly higher HBV DNA load compared to infected patients without MetS[13]. These results are not directly comparable as the our study used clinically diagnosed MetS compared to ultrasonographically diagnosed liver steatosis.

The association between HBV viral load and components of MetS is also interesting. Body weight, glycemia and triglycerides have typical distribution with pathological values on both ends of the spectrum. Authors of a cross-sectional study from Taiwan reported that the patients with BMI from 23 to 24.9 had the lowest levels of HBV DNA[53]. Analogous results have been produced by our study regarding total cholesterol levels and apolipoprotein B100 levels. Both parameters had quadratic relationship with HBV DNA load. Patients with subnormal but also higher than normal levels of total cholesterol and also apolipoprotein B100 had higher levels of HBV DNA compared to patients with both parameters in the normal range(Figure 1)[13]. Patients with low levels of total cholesterol often have advanced fibrosis that already impacts liver function. It is known that the risk of advanced fibrosis increases with the viral load[48]. On the other end of the spectrum, patients with high total cholesterol often have MetS and, as shown in our study[13], also the higher risk of increased HBV DNA. Other studies most commonly described linear inverse relationship of HBV DNA and other parameters of lipoprotein metabolism, such as high density lipoprotein[54] or triglycerides[21] that was not present in our data[13,14]. No relationship was revealed between HBV DNA and glycemia, HOMA-IR in any of the studies[21]. In the REVEAL study, HBV DNA load showed inverse correlation with extreme ($P = 0.004$) and central obesity ($P = 0.004$) even after adjustment

for triglycerides, hyperuricemia, gender and history of hypertension in HBe positive patients (*i.e.*, patients with high viral replication). In HBe negative patients inverse correlation with triglycerides was demonstrated[55].

CHB AND THE RISK OF ATHEROSCLEROSIS

CHB is an inflammatory condition. Other diseases with chronic low grade inflammation have been shown to increase the risk of atherosclerosis[56]. Nevertheless, the additional risk for atherosclerosis that could be attributed to CHB has been explored in surprisingly few studies. One study from Japan did not find significant differences between CHB patients and controls in systolic blood pressure, bilateral ankle brachial index, heart-ankle pulse wave velocity and the heart-carotid pulse wave velocity[57]. Also no significant difference was found when carotis intima-media thickness, maximal common carotid artery intima media thickness or extracranial carotid artery atherosclerotic score were evaluated in CHB patients[58]. Patients with HBsAg, but also antiHBc positivity have similar coronarography findings as uninfected, otherwise healthy patients[59,60]. No differences between these two groups of patients were found in the levels of high sensitivity C-reactive protein[60]. Despite these findings, it is necessary to note that CHB patients had significantly lower carotis intima-media thickness compared to the patients with NASH in one study[61]. Regarding clinical outcomes, patients with CHB had comparable cardiovascular mortality risk as uninfected patients[23].

It may seem that CHB patients do not have increased risk of atherosclerosis compared to uninfected patients, however several observations and hypotheses about the possible pro-atherogenic influence of CHB do exist. One paper from Turkey showed that inactive HBsAg carriers have greater mean platelet volume, considered to be an emerging risk factor for atherothrombosis, although clinical relevance of this observation is unknown[62]. Furthermore, a case report of 34 years old, hepatitis B infected man with multiple cerebral arterial stenoses without any risk factors for atherosclerosis was described by Korean authors[63].

MetS AND THE RISK OF FIBROSIS AND HCC IN PATIENTS WITH CHB

Both CHB as well as NAFLD associated with MetS led to the development of liver fibrosis, cirrhosis and HCC. Larger waist circumference, dyslipidemia and arterial hypertension in patients with CHB were associated with superimposed NASH in multivariate analysis[64]. A group of Spanish authors evaluated the severity of liver fibrosis by transient elastography in chronic HBV carriers, that were thought to be inactive. Central obesity, elevated fasting glucose, elevated TG, and lower HDL-C were associated with liver fibrosis[65]. Simultaneous presence of MetS and CHB increases the risk of liver fibrosis independently of biochemical activity and HBV DNA load[66]. CHB patients with MetS have higher prevalence of cirrhosis compared to CHB patients without MetS (38% *vs* 11%, $P < 0.001$)[67]. Kaplan-Meier analysis showed significantly more frequent development of cirrhosis and cirrhosis decompensation in CHB patients with diabetes mellitus compared to nondiabetic patients during 12-year follow-up[68]. Analysis of NHANES Ⅲ cohort showed that the presence of T2DM or insulin resistance is an independent predictor of mortality in CHB patients[69]. Another prospective study from Taiwan included 2903 HBsAg positive men followed for the median of 14.7 years. Higher BMI at baseline correlated with the incidence of NAFLD, liver cirrhosis and HCC. Higher BMI was also a significant risk factor for liver related mortality[70]. Body mass index, levels of insulin, glycated albumin and HOMA-IR correlated directly with the incidence of HCC, but triglycerides and LDL showed an inverse correlation[71]. CHB and MetS increased the risk of HCC, but also intrahepatic cholangiocarcinoma in United States population as well[72].

Because of the adverse influence of MetS and increased total cholesterol on the HBV DNA viral load and clinical outcomes of these patients, intervention with statin therapy has been proposed. Statins decrease HCV RNA load in patients with chronic hepatitis C[73,74]. Limited data are available on the pleiotropic effects of statins in patients with hepatitis B. In one study, the inhibition of HBsAg secretion into culture medium of Hep3B cells by lovastatin was observed[75]. Simvastatin has been shown to potentiate the anti-HBV activity of several nucleot(s)ide analogues (lamivudine, adefovir, tenofovir and entecavir) *in vitro*[76]. Data on clinical outcomes of statin therapy in CHB patients are even more limited. Recent study from Taiwan showed that this therapy reduces the risk of HCC in CHB patients in dose dependent manner[77]. Another observational study showed that CHB patients taking metformin or statins had lower risk of HCC as well[78]. Unfortunately, both trials were observational and no randomized controlled trials are available.

CONCLUSION

Multiple, but not all, studies showed that patients with CHB have lower risk of MetS, NAFLD and dyslipidemia. Patients with CHB without cirrhosis do not have increased risk of T2DM. CHB is probably not associated with higher risk of atherosclerosis as well. Regarding the clinical outcomes, available data do not sufficiently reveal all of the possible interactions between MetS, its individual components and CHB. Although more studies on the topic are needed, we can be reasonably sure that the simultaneous presence of both diseases accelerates fibrogenesis, increases the risk of liver

cirrhosis and HCC. Therefore, it is necessary to influence the MetS by lifestyle interventions as well as pharmacotherapy. Preliminary observational studies suggested the beneficial effect of statins and insulin sensitizers on the risk of HCC.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Prophylactic managements of hepatitis B viral infection in liver transplantation

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Abstract

Liver transplantation (LT) is a considerably effective treatment for patients with end-stage hepatitis B virus (HBV)-related liver disease. However, HBV infection often recurs after LT without prophylaxis. Since the 1990s, the treatment for preventing HBV reinfection after LT has greatly progressed with the introduction of hepatitis B immunoglobulin (HBIG) and nucleos(t)ide analogues (NAs), resulting in improved patient survival. The combination therapy consisting of high-dose HBIG and lamivudine is highly efficacious for preventing the recurrence of HBV infection after LT and became the standard prophylaxis for HBV recurrence. However, mainly due to the high cost of HBIG treatment, an alternative protocol for reducing the dose and duration of HBIG has been evaluated. Currently, combination therapy using low-dose HBIG and NAs is considered as the most efficacious and cost-effective prophylaxis for post-LT HBV reinfection. Recently, NA monotherapy and withdrawal of HBIG from combination therapy, along with the development of new, potent high genetic barrier NAs, have provided promising efficacy, especially for low-risk recipients. This review summarizes the prophylactic protocol and their efficacy including prophylaxis of *de novo* HBV infection from anti-HBc antibody-positive donors. In addition, challenging approaches such as discontinuation of all prophylaxis and active immunity through hepatitis B vaccination are discussed.

Key words: Liver transplantation; Hepatitis B infection; Prophylaxis; Nucleos(t)ide analogue; Anti-hepatitis B immunoglobulin

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Core tip: Combination therapy consisting of high-dose hepatitis B immunoglobulin (HBIG) and lamivudine has been the standard prophylaxis for hepatitis B virus recurrence after liver transplantation. Currently, after development of more potent high genetic barrier nucleos(t)ide analogues (hgbNAs), such as entecavir and tenofovir disoproxil fumarate, combination therapy using low-dose HBIG and hgbNA is considered as the most efficacious and cost-effective prophylaxis. In addition, monotherapy with hgbNAs and withdrawal of HBIG following combination therapy of HBIG and hgbNAs could be promising approaches, especially for low-risk patients and those receiving grafts from hepatitis B core antibody-positive donors. This review discusses those approaches including other challenging therapeutic options.

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INTRODUCTION

Liver transplantation (LT) is a highly effective treatment for patients with cirrhosis, liver cancer, or fulminant hepatitis caused by hepatitis B virus (HBV). However, the recurrence of HBV infection and subsequent severe disease, including an atypical, aggressive HBV recurrence pattern known as fibrotic cholestatic hepatitis (FCH), is a concern for patients with HBV-related diseases. In an era without effective prophylaxis, LT for HBV-related disease was a relative contraindication by the mid-1990s. Samuel *et al*[1] reported that the rate of HBV recurrence was 67% ± 4% among LT recipients with HBV-related cirrhosis and 83% ± 6% among those with positive post-LT serum HBV-DNA in the absence of any prophylaxis.

Several prophylaxis strategies for recurrent HBV have greatly progressed since the mid-1990s with the use of anti-hepatitis B immunoglobulin (HBIG) and nucleos(t)ide analogues (NAs). Therefore, LT outcomes for HBV-related diseases have markedly improved. Currently, combination therapy consisting of HBIG and NAs is widely used in most liver transplant centers. However, various issues exist with long-term combination therapy with HBIG and NAs after transplantation. In this article, we review present and future possible strategies, based on the previous history of prophylaxis, for preventing recurrent post-LT HBV infection. These include vaccine strategies that involve active immunization after LT.

INDICATIONS FOR LT AND PREOPERATIVE ANTI-VIRAL THERAPY FOR PATIENTS WITH HEPATITIS B AND RISK FACTORS FOR HBV RECURRENCE

LT is indicated for patients with irreversible hepatic failure due to HBV infection, which occurs through acute exacerbation of chronic hepatitis, cirrhosis, or fulminant hepatitis. The Child-Pugh score is widely used to evaluate the severity of cirrhosis. The one-year survival rate is 100% for patients with a Child-Pugh score of less than 6 points (Class A), while it is as poor as 50% for patients with a Child-Pugh score of 10 points or more (Class C). If a patient's clinical severity falls into Grade B or C (a score of 7 or above), LT may be required. The one-year survival rate after LT is as high as 80% to 90%, even for patients with Child-Pugh scores of 8 points or more. The model for end-stage liver disease (MELD) score is useful for predicting the short-term prognosis of patients with cirrhosis[2]. When the MELD score is below 14 points, patients have limited benefits from LT[3], while the risk of the transplantation itself increases when the score is too high. Thus, transplantation is considered optimal when the MELD score is above 15 points, but not after it becomes too high. In practice, the indications for transplant will be comprehensively determined by the patient's activities of daily living, complications, and the presence or absence of liver cancer or infectious diseases, among other indicators. In patients with HBV-related disease, several factors are considered as risks for HBV recurrence after LT[4-7]. High-risk patients include those who are hepatitis B e antigen (HBeAg) positive, as well as those who have high serum HBV-DNA levels at LT and anti-viral drug-resistance before LT[6]. On the other hand, low-risk patients include those with fulminant HBV, co-infection with hepatitis D virus[1], and low/negative serum HBV-DNA levels, which occur due to spontaneous or therapeutic features[8]. Among them, high serum HBV-DNA level at LT is a common significant risk factor that occurs regardless of protocol and is reported by many groups. Therefore, it is important to clear the virus from the blood or to reduce levels as much as possible by administering NAs before transplantation. Entecavir (ETV) and tenofovir disoproxil fumarate (TDF) are new potent agents that are recommended for the first-line treatment of NA-naïve patients due to low rates of drug resistance. For patients with renal disorders, which is often complicated by cirrhosis, ETV might be preferable compared to TDF because of the risk for nephrotoxicity. Currently, there are several

reports of patients developing resistant strains, such as the YMDD motif mutation, following long-term administration of lamivudine (LAM) among patients who are being considered for transplantation[9-11]. However, there is cross-resistance between LAM and ETV because a resistant mutation of LAM (*e.g.*, rtM204V/I and rtL180M) is also necessary in order to develop resistance to ETV[12]. In fact, many patients with LAM resistance have already acquired cross-resistance to ETV[13]. Therefore, ETV should not be used in patients with LAM resistance[14,15]. In this case, adding adefovir (ADV) or TDF to LAM, or switching to TDF but not ETV, is recommended. It should be noted that possible severe lactic acidosis might occur when ETV is used in patients with decompensated cirrhosis[16]. Interestingly, hepatocellular carcinoma (HCC) is believed to be a risk factor for HBV recurrence[17-19]. Saab *et al*[18] reported that 12 out of 175 patients (6.9%) developed HBV reinfection after LT despite combination therapy with HBIG and NAs. Among the 12 patients with HBV reinfection, 10 (83.3%) had HCC prior to LT and 5 (50%) developed HCC recurrence after LT[18]. Multivariate analyses revealed that pre-LT HCC and post-LT recurrence of HCC were independent risk factors for post-LT HBV reinfection. Although the mechanism remains unknown, close monitoring of virological status in patients with HCC is important.

PROPHYLAXIS OF RECURRENT HEPATITIS B IN HBSAG-POSITIVE RECIPIENTS AFTER LT

Monotherapy with HBIG or LAM

Prophylaxis of recurrent HBV infection with HBIG or LAM monotherapy has been performed since the 1990s for HBsAg-positive recipients. Several breakthrough studies demonstrated a reduction in reinfection and improvement of graft survival using HBIG[1,5,20,21]. Moreover, high-dose and long-term HBIG administration was shown to be most effective[1,20,21]. After the development of LAM, several groups investigated its use as a monotherapy without HBIG[22-25]. Perrillo *et al*[24] described the results of a multicenter US-Canadian trial where 25 out of 42 (60%) transplanted patients were HBsAg-negative at least three months post-transplantation. Patients also exhibited superior survival rates in comparison to a historical control group, which were similar to patients who were treated with long-term HBIG monotherapy[24]. However, monotherapy with either of HBIG or LAM resulted in the recurrence of HBV infection in 30% to 40% of patients, which was still a considerable rate despite the fact that graft survival greatly improved in most studies[1,21-24]. It is well known that amino-acid mutations in the predominant epitope of the HBs-Ag results in lower-binding affinity of anti-HBs Ab (*i.e.*, escape mutation). A significant correlation has been reported between the duration of HBIG monotherapy and the development of such mutants, indicating that mutations of the *S* gene were induced or selected by immune pressures exerted by HBIG[26]. Currently, long-term HBIG monotherapy is outmoded and not recommended for prophylaxis.

Combination therapy with HBIG and NAs

Following monotherapy with HBIG or LAM, a group from the University of California, Los Angeles, reported a landmark trial of combination therapy involving high-dose HBIG and LAM[27]. First described in 1998, this therapy successfully prevented the recurrence of HBV infection in almost all patients. In this trial, 14 patients who underwent LT for HBV-related decompensated liver disease received high-dose HBIG intra- and post-operatively in combination with pre- and post-operative LAM therapy as prophylaxis. Using polymerase chain reaction (PCR), all 13 surviving patients did not have detectable serum HBV-DNA at a median of 346 d (130-525 d) following LT. Similar high efficacy was demonstrated by other groups using this strategy[28,29]. Therefore, combination therapy consisting of high-dose HBIG and LAM became the gold standard for treatment and was rapidly introduced in many liver transplant centers. A recent meta-analysis revealed that combination therapy was superior in preventing reactivation of the virus when compared with monotherapy using HBIG or LAM alone[30].

Combination therapy with low-dose HBIG and NAs, and withdrawal of HBIG

Although the combination therapy consisting of high-dose intravenous HBIG and NAs is very effective, it is very expensive. Additionally, HIBG is not available in some countries. The first year and subsequent yearly treatment costs more than United States $100000 and United States $50000[31], respectively. In cost-effectiveness analysis, the cost-effectiveness is most sensitive to the cost of HBIG[32]. Therefore, optimizing the protocol by reducing the dose and duration of HBIG has been previously attempted. This concept is utilized in two strategies. One involves combination therapy with low-dose intramuscular HBIG and LAM, while the other includes the withdrawal of HBIG from combination therapy[33]. There are two more options in the combination therapy with low-dose HBIG involving either fixed low-dose or on-demand low-dose HBIG protocols.

For the fixed-dose protocol, Angus *et al*[34] showed that the combination of low-dose HBIG and LAM provides effective prophylaxis for post-LT HBV recurrence (Table 1). In this study, 400 or 800 IU of HBIG was administered intramuscularly (im) daily for 1 wk after transplantation and monthly thereafter, in combination with daily LAM (100 mg). Only 1 out of 32 patients was HBsAg positive and all 32 patients were

Table 1 Main results of published studies using combination therapy of low-dose hepatitis B immunoglobulin and nucleos(t)ide analogues and withdrawal of hepatitis B immunoglobulin from combination therapy

Ref.	Patients (*n*)	Median follow-up (mo)	NAs (dose)	HBIG protocol	HBV recurrence
Angus *et al*[34], 2000	32	18.4	32 LAM (100 mg/d)	400 IU or 800 IU/d im for 1 wk and then 400 IU or 800 IU/mo	1/32 (3.1%) HBsAg+ 0/32 (0%) HBV-DNA+
Gane *et al*[35],2007	147	62	147 LAM (100 mg/d)	400 IU or 800 IU/d im for 1 wk and then 400 IU or 800 IU/mo	5/147 (3.4%) actuarial risk of HBV recurrence was 1% at 1 yr and 4% at 5 yr
Karademir *et al*[36], 2006	35	16	33 LAM 2 LAM + ADV	6000 IU im intraoperatively, 2000 IU/d until HBsAb > 200 IU/L, and then 1200 to 2000 IU im on-demand if HBsAb < 100 IU/L, thereafter	2/35 (5.7%) Two HBV recurrent case had LAM resistance at LT
Iacob *et al*[38], 2008	42	21.6	42 LAM	10000 IU im in anhepatic phase and 10000 IU/d im for first 1 wk, and then 2500 IU im on-demand if HBsAb < 50 IU/L, thereafter	2/48 (4.8%)
Jiang *et al*[37], 2010	254	41.2	254 LAM	2000 IU im in anhepatic phase, followed by 800 IU/d for the next 6 d and weekly for the rest of 3 wk in the first postoperative month and 800 IU monthly or biweekly im on-demand if HBsAb < 100 IU/L, thereafter	14/254 (5.5%) The 1-, 3- and 5-yr HBV recurrence rates were 2.3%, 6.2% and 8.2% 5 of 14 recurrent cases had YMDD mutants at recurrence
Nath *et al*[41], 2006	14	14.1	14 LAM + ADV	10000 IU HBIG iv in anhepatic phase and 10000 IU/d for first 1 wk, and then HBIG was withdrawn and replaced with oral ADV	1/14 (7.1%) HBV recurrent case showed normal liver function.
Angus *et al*[42], 2008	34	21	18 LAM + HBIG 16 LAM to LAM + ADV	Randomized trial All patients were treated with low-dose im HBIG + LMV ⩾ 1 yr post-LT 18 patients continued HBIG *vs* 16 patients discontinued HBIG and ADV was added (LMV + ADV)	0/18 in HBIG + LMV 1/16 (6.3%) in LMV + ADV (HBIG withdrawal group) Recurrent case was HBsAg+/HBV-DNA-
Saab *et al*[43], 2011	61	15	19 LAM to LAM + ADV 41 LAM to LAM + TFV 1 ETV to ETV + ADV	All patients were treated with low-dose im HBIG + LMV ⩾ 1 yr post-LT. All patients discontinued HBIG, and ADV or TDF was added as described left	2/61 (3.3%) Both recurrent case was HBsAg+/HBV-DNA- without liver dysfunction

HBV: Hepatitis B virus; NAs: Nucleos(t)ide analogues; HBIG: Hepatitis B immunoglobulin; LT: Liver transplantation; HBsAb: Hepatitis B surface antibody; LAM: Lamivudine; ADV: Adefovir; ETV: Entecavir; TDF: Tenofovir disoproxil fumarate; im: Intramuscularly.

HBV DNA negative at the latest follow-up visit (mean follow-up period, 18.4 mo). Subsequently, Gane *et al*[35] reported the long-term results of an expanded population, including high-risk patients from the same group of HBV-DNA positive patients, at LT (85%) (Table 1). One hundred and forty-seven patients were analyzed and the actuarial risk of HBV recurrence was 1% and 5% after 1 and 5 years, respectively. The high HBV-DNA titer at LT was associated with HBV recurrence.

Karademir *et al*[36] previously reported on the efficacy of the on-demand protocol (Table 1). Patients received 6000 IU intra-operatively, 2000 IU daily, and 2000 IU as on-demand maintenance medication for life, respectively, in order to keep the serum level at 100 IU/L. Two of the 35 patients who had experienced HBV recurrence (5.7%) after a median follow-up of 16 mo were HBV-DNA positive at the time of LT, despite preoperative LAM administration due to LAM resistance. The mean cumulative dose of HBIG, that was administered within the first, second, and third years were 34014, 5258, and 5090 IU, respectively. Jiang *et al*[37] evaluated the low-dose, on-demand protocol in a larger population (Table 1). Two hundred and fifty-four adult patients received LAM (100 mg/d orally) and HBIG (2000 IU, im) in the anhepatic phase, which was followed by 800 IU daily for the next 6 d, and weekly for the rest of 3 wk in the first postoperative month. This was followed by the administration of 800 IU monthly or biweekly, in order to maintain hepatitis B surface antibody (HBsAb) levels at 100 IU/L. Their 1-, 3-, and 5-year HBV recurrence rates were 2.3%, 6.2% and 8.2%, respectively, after a mean follow-up period of 41.2 mo. Fourteen patients experienced post-transplant HBV recurrence. High pre-transplant HBV-DNA levels (> 10^5 copies/mL) at LT and post-transplant prednisone withdrawal times (> 3 mo) were associated with recurrences. Regarding the optimal target HBsAb titer, Iacob *et al*[38] reported successful prophylaxis for 42 patients with lower HBsAb maintenance levels (10000 IU within the anhepatic phase and daily within the first postoperative week, followed by 2500 IU on demand if HBsAb titers fell below 50 IU/L) (Table 1). The HBV recurrence rate was 4.8% with a median follow-up period of 1.8 years. Currently, the optimal target

HBsAb titer of 50-100 IU/L is most accepted, although it depends on the involved institutions. This low-dose, on-demand protocol was also effective in living donor liver transplantation[39,40].

The withdrawal of HBIG from combination therapy is another strategy that was reported by several groups. Nath *et al*[41] evaluated the efficacy of this protocol. Fourteen patients with HBV-related disease received intravenous HBIG (10000 IU) administration during the anhepatic phase at LT, and daily for 7 d in combination with LAM. HBIG was subsequently discontinued and replaced with the oral administration of ADV. One patient experienced an HBV recurrence (7.1%) with a mean follow-up of 14.1 mo. Angus *et al*[42] reported a multicenter randomized study of ADV substitution for low-dose intramuscular HBIG. In this study, 34 patients without HBV recurrences at least 12 mo post-LT were randomized to either the ADV replacement (16) and HBIG continuation (18) groups. Although one patient in the ADV group (6.2%) had a HBV recurrence, HBV-DNA was undetectable in the subsequent 20 mo. Saab *et al*[43] also reported low recurrence rates (3.3%) in 61 patients who changed their regimen from low-dose HBIG and nucleoside analogue therapy to nucleoside and nucleotide analogue therapies, respectively.

Both Combination therapy consisting of low-dose intramuscular HBIG and NAs and the withdrawal of HBIG, were more cost-effective compared to the combination therapy consisting of high-dose, indefinite HBIG, and NAs.

In most studies that utilized the HBIG withdrawal protocol, HBIG was replaced with a nucleotide analogue (mostly ADV) in combination with LAM and this likely resulted in favorable outcomes. Furthermore, it has been reported that HBV recurrence was associated with low HBIG dose during the first week post-LT in patients receiving low-dose HBIG and LAM suggesting that perioperative, high-dose HBIG administration might compliment the low efficacy of LAM[44]. These facts would also imply that usage of new and emerging, more potent high genetic barrier NAs (hgbNAs) such as ETV or TDF could increase the efficacy of these protocols. In fact, in the combination therapy of low-dose HBIG and NAs, ETV has been recognized as superior to LAM[44-46]. In their systematic review of 519 HBV liver transplant recipients from 17 studies, Cholongitas *et al*[44] recently indicated that patients who were treated with HBIG and LAM developed HBV recurrence significantly more frequently, compared to patients who were treated with HBIG and ETV or the TDF combination (6.1% *vs* 1.0%, $P < 0.001$). They also showed that hgbNA administration, along with HBIG discontinuation, was not inferior to the combination of HBIG and hgbNA [3.9% (4/102) *vs* 1.0% (3/303), $P = 0.17$]. Furthermore, it has been reported that HBIG dose and duration had no impact on HBV recurrence rate if combined with hgbNAs (ETV or TDF)[7]. This result might support the concept of NAs monotherapy for HBV prophylaxis, as described in following section.

Prophylaxis without HBIG (NA monotherapy)

As mentioned above, LAM monotherapy without HBIG after LT is partially effective but is not sufficient as prophylaxis for recurrent HBV infection[22-24]. Therefore, it has been rapidly replaced with the combination therapy involving HBIG and LAM/other NAs.

However, along with development of more potent hgbNAs such as ETV or TDF, the necessity of HBIG for prophylaxis and efficacy of monotherapy with hgbNAs has been the subject of much discussion. Four reports, including three retrospective cohort studies[47-49] and a prospective cohort study[50] on hgbNA monotherapy have been published to date. Cholongitas *et al*[46] conducted a systemic review of 112 patients who received hgbNAs without HBIG prophylaxis. Cholongitas *et al*[46] showed that the post-LT HBV recurrence rate was higher in patients with mono-prophylaxis using hgbNAs than in those who were taking a combination of HBIG and LAM [26% (29/112) *vs* 5.9% (109/1834), $P < 0.001$] when HBV recurrence was defined as being HBsAg positive. However, HBV-DNA was detectable in only one patient in the mono-prophylaxis group and the recurrence rates in both groups were comparable (0.9% *vs* 3.8%, $P = 0.11$) when HBV recurrence was defined as the presence of serum HBV-DNA.

More recently, Gane *et al*[51] reported that the combination of LAM and ADV, without HBIG was a safe and effective prophylaxis for post-LT HBV recurrence. Eighteen patients with pre-LT serum HBV-DNA (< 3 $\log_{10}$ IU/mL) suppression were selected to receive HBIG-free prophylaxis with LAM and ADV and did not show any HBV recurrences after a median post-LT follow-up of 22 mo. Fung *et al*[47] reported the long-term outcomes of 362 patients who were receiving post-LT NA monotherapy without HBIG. The authors showed that the virological relapse rates at 3 years for the LAM, ETV, and combination groups (predominantly LAM + ADV) were 17%, 0% and 7%, respectively ($P < 0.001$). The majority of patients with virological relapses were in the LAM and combination groups and had the YMDD mutation. No resistance mutations were identified in the ETV group. These results imply that NA monotherapy without HBIG using either single potent hgbNA or the combination of nucleoside and nucleotide analogues, which have been associated with a very low rate of virologic breakthroughs, might suffice in the prophylaxis of HBV infections. In the current era, increasing the use of potent NAs before LT and NA monotherapy for post-LT prophylaxis is expected. The studies described above suggest the satisfactory and promising prophylactic efficacy of hgbNA monotherapy. Nonetheless, the preoperative administration of hgbNAs in the current age can achieve HBV-DNA negativity in many LT candidates, and such low risk

patients were included in studies described above. Therefore, low-risk patients should be selected for NA monotherapy. Furthermore, the inconsistencies between the HBsAg and HBV-DNA appearances have also been seen in NA monotherapy. HBsAg-positive and HBV-DNA-negative patients receiving hgbNA monotherapy had normal graft function without any sign of hepatitis. Long-term, larger, and randomized studies are needed in order to re-evaluate the clinical impact of HBsAg and HBV-DNA levels in this setting.

Interestingly, it has been reported that prophylactic HBIG prevents acute rejection after LT[52-54]. Kwekkeboom *et al*[52] reported that recipients receiving HBsAg-positive liver grafts and treated with prophylactic HBIG (n = 40) showed a significantly lower incidence of acute rejection compared to recipients without viral hepatitis (n = 147) (12% *vs* 34%, P = 0.012). Furthermore, HBIG suppressed functional maturation of human blood-derived dendritic cells (DCs) and cytokine production as well as alloantigen- and lectin-stimulated peripheral T cell proliferation *in vitro* at concentrations similar to that during HBIG treatment. These findings suggest that HBIG still has a significant role as an allo-immunosuppressant as well as a prophylactic agent.

Discontinuation of all prophylaxis

How long a prophylactic treatment should be continued has been previously debated. Cheung *et al*[55] reported occult post-LT HBV infection of both donor and recipient origins despite NA prophylaxis. In this study, 31 patients who received post-transplant NAs prophylaxis remained seronegative for HBsAg for a median of 44.5 mo. Nineteen of these recipients (61%) had received anti-HBcAb positive grafts. Intrahepatic total HBV DNA and levels were quantified, and the sequence was analyzed. Intrahepatic total HBV DNA and covalently closed circular DNA were detected in 26 (84%) and 16 (52%) of the 31 recipients, respectively, although none of them had detectable serum HBsAg and HBV-DNA. A phylogenetic analysis of the isolated HBV DNA sequence revealed HBV infections of both donor and recipient origins. These results showed that an occult HBV infection after LT from either the recipient or donor could be present despite the absence of serum HBsAg and HBV-DNA after prophylaxis. Lifelong prophylaxis is advocated based on these data. Lenci *et al*[56] have recently addressed this issue. They investigated the safety of withdrawal of all HBV prophylaxis regimens in selected LT recipients according to a stepwise approach. After receiving a combination of HBIG with LAM (± ADV) as prophylaxis, 30 low-risk patients (*i.e.*, HBeAg and HBV-DNA negative at LT) underwent sequential liver biopsies in order to confirm the absence of intrahepatic total and cccDNA. Based on biopsy results, HBIG and then NAs were withdrawn sequentially. Twenty-five patients did not showed clinical signs of HBV recurrence after withdrawal of prophylaxis with a median follow-up period of 28.7 mo, while 5 patients subsequently became HBsAg-positive. All 25 patients who did not have recurrences did not have detectable total/ccc DNA in their liver specimens, while the 5 patients with recurrences had detectable total HBV-DNA in their tissues. This study suggests that a sensitive method using protocol biopsy might be useful for identifying candidates for complete withdrawal of prophylaxis. However, this concept remains challenging and larger studies with longer follow-up are necessary.

It is important to remember that HBV recurrence has been conventionally diagnosed by the persistence or reappearance of serum HBsAg and/or HBV-DNA. PCR assays have demonstrated that HBV-DNA is typically detected prior to HBsAg re-appearance[57]. However, in this study, 4 out of 5 patients who became HBsAg-positive did not have detectable HBV-DNA in their serum, or experience any clinically relevant events. This inconsistency between HBsAg and HBV-DNA appearances has also been seen in HBIG-free prophylaxis, as described above[46]. All HBs Ag positive and HBV-DNA negative patients receiving HBIG-free prophylaxis with hgbNAs had normal graft functions without any sign of hepatitis. These findings might indicate the necessity for redefining HBV reinfection. Its clinical impact should be confirmed in future studies.

Active immunity through hepatitis B vaccination

Although the virus is cleared after surgery by the aforementioned prophylaxis for HBV recurrence, hepatitis could recur if treatment is discontinued. Prolonging the treatment involves safety issues such as the development of escape mutations and/or emerging resistant strains due to long-term administration of HBIG and NAs, as well as economic issues[58]. These considerations are also important in the prevention of *de novo* HBV infection that is induced by anti-HBc antibody (HBcAb)-positive donors. Theoretically, the development of natural, long-lasting anti-HBsAb in the recipient through active immunotherapy with a vaccine is the most ideal approach in terms of cost and benefit, in order to avoid the issues that are encountered after LT and HBIG replacement. Nonetheless, this approach has not been used widely because of its low response rate in most trials (Table 2)[59-62]. This low response rate is caused by the difficulty in introducing active immunity under an immunosuppressed state after transplantation. Newer data suggest the enhancement of vaccination rates with the addition of more effective adjuvants or the administration of third generation recombinant vaccines. Some groups showed that HB vaccine plus more potent adjuvants such as monophosphoryl lipid A was much more effective for post-LT patients[63-65]. Another group has shown the efficacy of pre-S containing third generation vaccines[66]. However, as shown in Table 2, the long duration between LT, vaccination, and mono-immunosuppression, especially

Table 2 Main results of published studies using vaccination after liver transplantation

Ref.	Patients (*n*)	Age, median (yr)	HBV-DNA negative before LT	Duration between LT and vaccination	Momo immunosuppression	Response to vaccination[1]	Vaccination protocol (dose: μg)
Sánchez-Fueyo *et al*[59], 2000	22	39	100%	33%	63.5	22.7%	40 im/40 im 3 + 3 (/cycle) without HBIG
Angelico *et al*[60], 2002	17	53	100%	48%	100	11.8%	40 im/10 sc/40 im 3 + 6 + 3 (/cycle) without HBIG
Bienzle *et al*[63], 2003	20	54	100%	78%	80	80%	20 im or 100 im 5 + 3 (/cycle) with HBIG Adjuvant: MPL + QS21
Stärkel *et al*[64], 2005	10	49	90%	55%	100	40%	40 im/40 im 5 (/cycle) with HBIG Adjuvant: MPL
Lo *et al*[66], 2005	52	47	81%	14%	92.3	1.9%	40 im 3 + 3 (/cycle) without HBIG
Rosenau *et al*[62], 2006	8	50	37.5%	60%	37.5	12.5%	20 im 6 (/cycle) without HBIG
Lo *et al*[66], 2007	20	52	80%	21%	85	35%	HBs + preS 20 im 3 + 3 (/cycle) without HBIG
Tahara *et al*[69], 2009	20	53	75%	20%	60	65%	20 im or 40 im unrestraint with HBIG under immune-monitoring
Di Paolo *et al*[65], 2010	18	59	100%	73%	89	44%	20 im 6 + 6 (/cycle) HBIG withdraw Adjuvant: MPL

[1]Anti-HBsAb level ⩾ 100 IU/L. HBV: Hepatitis B virus; MPL: Monophosphoryl lipid A; HBIG: Hepatitis B immunoglobulin; QS21: Quillaja saponaria Molina; HBsAb: Hepatitis B surface antibody; LT: Liver transplantation; im: Intra-muscular administration; sc: Subcutaneous administration.

with withdrawal of steroids, seems to be necessary for vaccination to be effective. This suggests the significance of immune-optimization for successful treatment[59]. In our facility, we are trying to optimize immunosuppression by monitoring immune status using the CFSE-MLR (mixed lymphocyte reaction using carboxyfluorescein succinimidyl ester)[67,68], in order to achieve a more effective introduction to active immunity and the withdrawal of HBIG therapy through unrestricted HB vaccination. Using these methods, we have seen an increase in anti-HBsAb values in 13 out of 20 patients who received HB vaccination after LT (65%) and have had successful HBIG[69] withdrawal. When comparing the immunocompromised state during HB vaccination, the responder group was in the anti-donor-specific immunocompromised state, which maintained immunity against the third party, while the non-responder group was in a nonspecific immunocompromised state. Although a reduction in anti-HBsAb levels was observed after withdrawal of HBIG in 8 out of 13 successfully immunized cases, no re-administration of HBIG was required because the antibody levels increased again after short-term HB vaccine re-administration. According to our results, the proper maintenance of long-term immune status and administration of the HB vaccine for a lengthy period, if needed, is important for successful HB vaccination therapy. The establishment of standardized HB vaccination therapy is expected in the near future, along with the development of a more efficient novel hepatitis B vaccine, although additional large studies will be needed prior to universally recommending this strategy.

PROPHYLAXIS OF *DE NOVO* HBV INFECTION FROM HBCAB-POSITIVE DONORS

Transplantation from HBcAb-positive donors to HBV-uninfected recipients is clinically critical. Ideally, in order to prevent HBV transmission, HBcAb-positive donors should not be accepted. Since a considerable percentage of the population is HBcAb-positive and in

the presence of a chronic and global organ shortage, the use of HBcAb-positive donor livers would expand the donor pool. Nonetheless, *de novo* HBV infection would be a problem, particularly with the combination of HBcAb-positive donor and HBcAb- and HBsAg-negative recipients[70,71], respectively. Dickson *et al*[70] evaluated 674 LT patients and reported that *de novo* HBV infection developed in 18 out of 23 recipients of livers from HBcAb-positive donors (78%) compared with only 3 (0.5%) out of 651 recipients of HBcAb-negative donor livers ($P < 0.0001$). Furthermore, LT from HBcAb-positive donors were associated with a decreased four-year survival rate (adjusted mortality hazard ratio of 2.4; 95%CI: 1.4-4.0). Therefore, the establishment of necessary and sufficient prophylaxis for *de novo* HBV infection is an important area for therapeutics in LT cases. An adequate consensus has not been established and prophylactic strategies that are currently used for LT from HBcAb-positive donors vary from the administration of HBIG or NAs alone to combination therapy, depending on the liver transplant centers. Several groups studied the efficacy of HBIG monotherapy for the prophylaxis of *de novo* HBV infection in recipients who received grafts from HBcAb-positive donors. Roche *et al*[72] showed that HBV infections were observed in 31.6% of HBV-naïve recipients despite HBIG monotherapy. Furthermore, HBIG monotherapy was associated with a significant risk of *de novo* HBV infection and escape mutation. Ueda *et al*[58] retrospectively analyzed the clinical course of 75 patients who received HBIG prophylaxis for greater than 6 mo after LT with HBcAb-positive donor grafts. In this study, 19 out of 75 patients (25%) developed *de novo* HBV infection and escape mutations were detected in 7 out of 19 patients[58]. Conversely, Yu *et al*[73] showed the excellent prophylactic effect of LAM monotherapy for HBsAg-negative patients receiving HBcAb-positive donor grafts (0 out of 9 patients) with no evidence of HBV-DNA in the grafts. Two systematic reviews confirmed these results[74,75]. Cholongitas *et al*[74] reported *de novo* HBV infection rates of 19%, 2.6% and 2.8% in HBsAg-negative recipients treated with HBIG, LAM, and their combination, respectively, in their systemic review. These data indicate the sufficient prophylactic potential of LAM monotherapy and the absence of supplementary effects of HBIG for preventing de novo HBV infection. However, the necessity of lifelong prophylaxis needs to be elucidated in future long-term studies.

CONCLUSION

LT for patients with hepatitis B has been one of the most successful treatments in the LT field for decades because of several effective prophylaxis treatments, especially the development of potent hgbNAs. Currently, combination therapy using low-dose HBIG and hgbNA is likely the most accepted prophylaxis. Monotherapy with hgbNAs and withdrawal of HBIG following combination therapy with HBIG and hgbNAs, are promising approaches.

FUTURE PERSPECTIVES

Treatment strategies that involve hgbNA monotherapy or the withdrawal of HBIG are effective and would be accepted in more institutions. However, further studies are required to decide appropriate regimens (timing and duration) and select optimal patient subpopulations for those strategies. Long-term compliance with the administration of anti-HBV drugs including hgbNAs and newly emerging drug resistance are other issues that need to be addressed. Therefore, close monitoring of recipients with NAs prophylaxis is important. Active immunity through hepatitis B vaccination might be another promising measure for resolving these issues after a more effective and standardized hepatitis B vaccination therapy has been established.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Autophagy and microRNA in hepatitis B virus-related hepatocellular carcinoma

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Abstract

Approximately 350 million people worldwide are chronically infected by hepatitis B virus (HBV). HBV causes severe liver diseases including cirrhosis and hepatocellular carcinoma (HCC). In about 25% of affected patients, HBV infection proceeds to HCC. Therefore, the mechanisms by which HBV affects the host cell to promote viral replication and its pathogenesis have been the subject of intensive research efforts. Emerging evidence indicates that both autophagy and microRNAs (miRNAs) are involved in HBV replication and HBV-related hepatocarcinogenesis. In this review, we summarize how HBV induces autophagy, the role of autophagy in HBV infection, and HBV-related tumorigenesis. We further discuss the emerging roles of miRNAs in HBV infection and how HBV affects miRNAs biogenesis. The accumulating knowledge pertaining to autophagy and miRNAs in HBV replication and its pathogenesis may lead to the development of novel strategies against HBV infection and HBV-related HCC tumorigenesis.

Key words: Hepatitis B virus; Autophagy; MicroRNA; Hepatocellular carcinoma; Viral replication

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Core tip: A number of reviews have described autophagy and microRNAs (miRNAs) in the hepatitis B virus (HBV)-related tumorigenesis. However, few reviews have provided insights into the relationships among autophagy, miRNAs, HBV biogenesis and hepatocarcinogenesis. In this review, we describe

the emerging role of autophagy and miRNAs in HBV replication and pathogenesis. Recent studies are reviewed in the following sections: (1) the mechanism by which HBV induces autophagy; (2) the effect of HBV-induced autophagy on HBV replication; (3) the relationship between autophagy and HBV in HBV-related hepatocarcinogenesis; (4) the mechanism by which miRNAs affects HBV replication; and (5) the regulation of miRNAs biogenesis by HBV.

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INTRODUCTION

Proper autophagic responses protect the cell from stresses and maintain cellular homeostasis. Autophagy progression is regulated by a series of autophagy-related genes (*Atg*). *Atg5, Atg7* and *Beclin 1* are essential genes in the autophagic process and silencing any of these genes blocks autophagy[1]. A novel function of autophagy known as secretory autophagy (also called autosecretion or type Ⅲ secretion) has been the subject of increasing research interest[2]. In contrast to traditional degradative autophagy, the recruited cargo is carried by the double-membrane autophagosome and exported to the extracellular environment (exocytosis). Traditionally, the cargo in the cell is delivered through the endoplasmic reticulum-Golgi apparatus-plasma membrane pathway[3]. However, specific cargo proteins in the cytosol are transported to the extracellular environment without going through the endoplasmic reticulum-Golgi apparatus-plasma membrane pathway. This unconventional secretory autophagy has been shown to be involved in vesicle trafficking and cytokines secretion (IL-1β, IL-6, IL-18 and TNF-α) for innate and adaptive immune responses[4].

Cancer progression triggers diverse metabolic stresses which lead to increased autophagic activity. Aberrant autophagy may cause cell death which is known as type Ⅱ programmed cell death. Recent evidence indicates that autophagy suppresses tumorigenesis to preserve cellular fitness and genome integrity[5,6]. Therefore, manipulation of autophagic activity may have potential in the development of an alternative therapeutic strategy against cancer development or drug resistance in cancer cells[7-9]. Deficient autophagic responses cause diverse pathologic conditions of the liver, including liver dysfunction and tumorigenesis[9,10]. Autophagic machinery is also important for innate and adaptive immunity. In innate immunity, diverse pathogens including bacteria and viruses are selectively engulfed by the autophagosome followed by fusion with the lysosome to form the autolysosome and autolysosome-mediated clearance[11]. In adaptive immunity, autophagic machinery produces antigenic peptides, which are loaded onto the major histocompatibility complex (MHC) class Ⅱ molecules and presented to $CD4^+$ T cells[12]. Pathogen infection of the host cells causes cellular stress. To diminish the deleterious impact of stress, the autophagic degradation system is induced to recruit the damaging molecules including proteins, organelles, pathogens, and microRNAs, which are subsequently subjected to autophagic degradation. Meanwhile, pathogens utilize various strategies to escape, suppress or hijack the autophagic degradation pathway. They may also interfere with autophagy-related immune defenses[13,14].

MicroRNAs (miRNAs) are small non-coding RNAs which are initially transcribed as long primary miRNAs which then undergo sequential processing to form precursor miRNAs (pre-miRNA) by RNase Ⅲ endonucleases. Pre-miRNAs are then transported into the cytoplasm where they are transformed by Dicer processing to become mature miRNAs[15]. MiRNAs suppress their target-gene expression either by transcriptional degradation or by translational inhibition, depending on sequence homology between the miRNA and the target gene. MiRNAs are involved in diverse diseases including viral infections and cancers[16].

Hepatitis B virus (HBV) is characterized by partly double-stranded relaxed circular DNA (rcDNA) and belongs to the hepadnaviridae family. The HBV virion consists of an outer envelope and the inner core proteins, 3.2 kb of rcDNA genome and DNA polymerase[17]. During HBV infection of the hepatocytes, the uncoated HBV rcDNA is transported to the nucleus. In the presence of viral DNA polymerase, the rcDNA is transformed into covalently closed circular DNA (cccDNA), which serves as the template for transcription of viral mRNAs in the presence of host RNA polymerase. The HBV genome contains four overlapping open reading frames (ORFs) comprising the S, C, X and P regions. The S region encodes three envelope proteins (S, M, and L) for viral envelopment and the C region encodes the core protein for the viral capsid. The X region encodes the X protein for viral replication. The P region encodes the proteins for viral RNA reverse transcription and DNA replication[18]. The progeny nucleocapsid harboring the rcDNA then proceeds to viral envelopment and mature virus release. In this review, we conduct an in-depth exploration of the role of autophagy and miRNAs in HBV infection and pathogenesis.

AUTOPHAGY AND HBV

HBV infection can induce autophagy and different genotypes of HBV have shown different increments of autophagic activities[19]. The following sections summarize the underlying mechanisms of HBV-

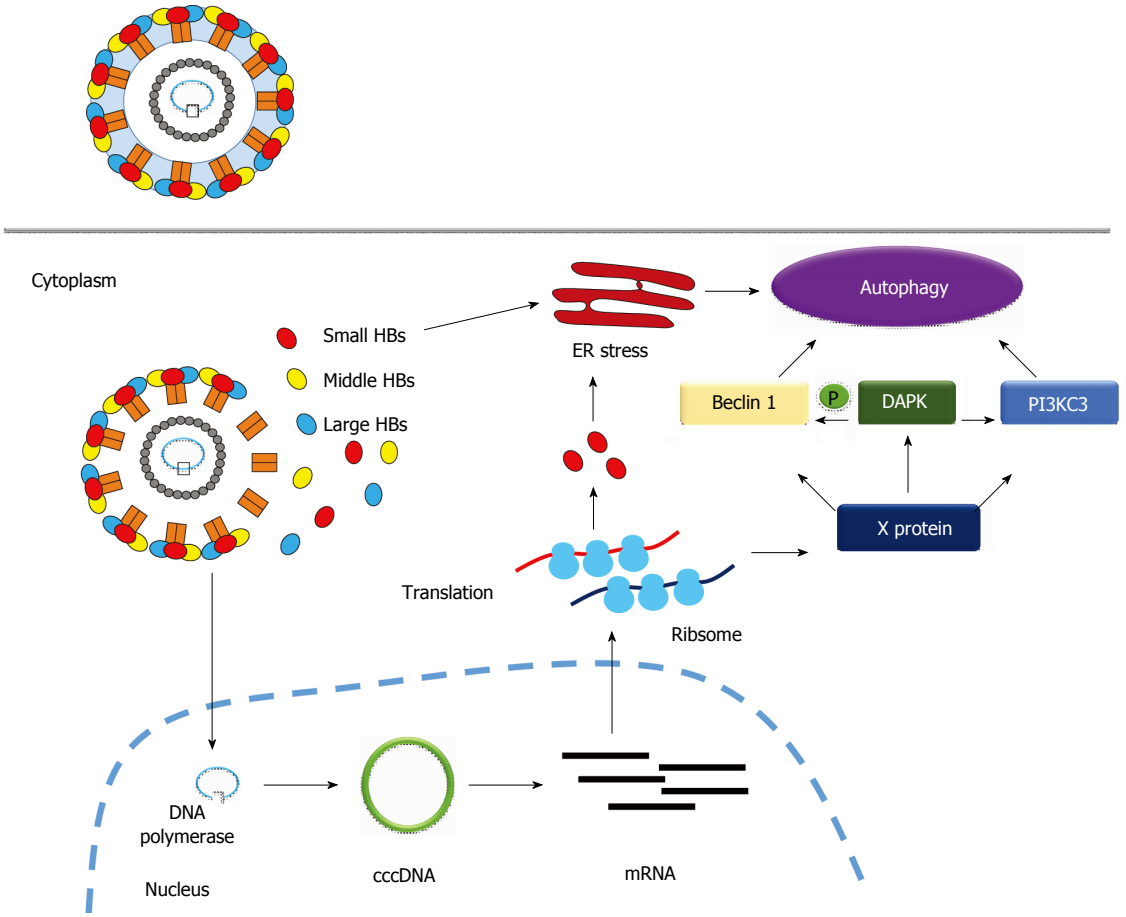

Figure 1 How hepatitis B virus infection induces autophagy. Hepatitis B virus (HBV) induces autophagy mainly through HBx protein or small surface protein (Small HBs)-related mechanism. The former (HBx) induces autophagy by the following routes: (1): Increases mRNA expression of *Beclin 1* through transcriptional regulation; (2): Activates enzymatic activity of PI3KC3 to enhance autophagosome formation; (3): Activates DAPK to trigger autophagy in a Beclin 1-PI3KC3-dependent manner. The latter (SHBs) triggers ER stress to induce autophagy. DAPK: Death-associated protein kinase.

induced autophagy and the roles of autophagy in HBV infection, replication, and HBV-related hepatocellular carcinogenesis.

HBV induces autophagy

HBV induces autophagy mainly through the HBx protein or small surface protein (SHB)-dependent mechanism.

HBx protein induces autophagy: The HBx protein has been demonstrated to be the major molecule involved in inducing autophagy during HBV infection[20-23]. Beclin 1 is an autophagy-related protein (the mammalian orthologue of yeast Atg6), which forms a complex with PI3KC3 to initiate autophagic progression. Beclin 1 is responsible for localization of the autophagic complex proteins (PI3KC3 and UVRAG) to the pre-autophagosomal structure[24]. AMP-activated protein kinase and mTORC1 signaling molecules, the sensors of nutrient and energy, regulate the Beclin 1-PI3KC3 complex to produce phosphatidylinositol 3-phosphate (PI3P), a signaling lipid for the recruitment of autophagy effectors[25]. HBx directly increases *Beclin 1* expression through the activation of the -277/+179 region of *Beclin 1* promoter, which further enhances starvation-induced autophagy (Figure 1)[20]. HBx can also increase the enzymatic activity of PI3KC3, which mediates PI3P formation and enhances autophagosome formation (Figure 1)[21]. Autophagy can be induced by death-associated protein kinase (DAPK) through phosphorylation of Beclin 1 and protein kinase D, which activates PI3KC3[26]. DAPK is a calcium/calmodulin serine/threonine kinase and is associated with different cell death pathways, including autophagic cell death[27]. DAPK phosphorylates Beclin 1 on the BH3 domain to promote its dissociation from Bcl-X_L, which triggers autophagic progression[28]. Zhang *et al*[23] demonstrated that HBx activates DAPK through dephosphorylation of DAPK, and induces autophagy in a Beclin 1-dependent manner (Figure 1). In summary, the HBx protein induces autophagy at the initiation stage of autophagic progression.

HBV small surface protein (SHB) induces autophagy: The HBV envelope proteins also plays a role in HBV-induced autophagy. There are three envelope proteins, including large, medium, and small surface proteins (SHBs), in HBV. Li *et al*[29]

found that deletion of the HBV envelope proteins abrogate HBV-induced autophagosome formation. They further demonstrated that the intracellular SHBs, but not extracellular SHBs, trigger endoplasmic reticulum (ER) stress and unfolding protein responses including ATF-6, PERK and IRE1 signaling pathways to induce autophagy (Figure 1). SHBs do not affect the expression level of Beclin 1, and therefore involves a different mechanism to that of HBx-induced autophagy. The phenomena described above indicate that HBV may use various structure proteins to induce autophagy through different mechanisms.

The effect of HBV-induced autophagy on HBV replication

HBV infection is thought to follow a particular sequence involving virus entry followed by cccDNA synthesis, mRNA transcription, viral protein synthesis, encapsidation, viral DNA replication (reverse transcription), envelopment and release of the mature viruses[30]. HBV-induced autophagy positively or negatively regulates virus replication at different stages of HBV infection.

Promotion of HBV replication - DNA replication: To clarify the effect of HBV-induced autophagy on HBV replication, Sir *et al*[21] suppressed autophagy using an inhibitor (3-MA) or by knocking out autophagy-related genes using siRNA (si-Atg7 or si-Vps34) during virus infection, and revealed that inhibition of autophagy only slightly decreased viral mRNA synthesis and HBV RNA packaging. HBV DNA replication was significantly suppressed, suggesting that autophagy mainly enhances HBV replication. They further revealed colocalization of the HBV core protein and autophagosome during HBV infection, suggesting that the autophagosome may function as the docking site for viral replication in a similar manner to that observed in poliovirus and dengue virus infection[31,32]. Tain *et al*[33] utilized the HBV transgenic mice harboring liver-specific knockout of *Atg5* gene (*Atg5*$^{-/-}$) to demonstrate that autophagy enhances HBV replication *in vivo*. In summary, these data indicate that autophagy promotes HBV replication through induction of viral DNA replication (Figure 2). Whether unconventional secretory autophagy plays a role in HBV replication and release warrants further investigation.

Promotion of HBV replication: viral envelopment: During HBV infection, viral DNA is synthesized through reverse transcription within the nucleocapsid, which then progresses to envelopment in the Golgi apparatus and becomes the mature virion followed by exocytosis to release the mature viruses from the cell. HBV nucleocapsid-associated DNA (Ⅰ), intracellular enveloped DNA (Ⅱ) and extracellular enveloped DNA (Ⅲ) represent the three steps of HBV viral nucleocapsid, envelopment, and secretion. Li *et al*[29] showed that suppression of HBV-induced autophagy slightly reduced nucleocapsid-associated DNA (Ⅱ) but significantly decreased intracellular enveloped DNA (Ⅱ) and extracellular enveloped DNA (Ⅲ), indicating that HBV-induced autophagy plays a major role during viral envelopment (Figure 2). Finally, colocalization of HBV envelope proteins with the autophagosome supports the notion that autophagosome may serve as the docking site for HBV envelopment.

Suppression of HBV replication - Degradation of envelope proteins: ER plays multiple functions including protein folding and transporting cargo to the Golgi apparatus. Misfolded proteins may accumulate, causing ER stress and unfolded protein responses (UPR)[34]. HBV infection induces ER stress and activation the IRE1-XBP1 signaling pathway of UPR. Accordingly, HBV envelope proteins are translocated into ER for viral envelopment[35,36]. To relieve HBV-induced UPR, EDEM (ER degradation-enhancing, mannosidase-like) proteins recognize and transport the misfolded glycoproteins to the ER-related signaling pathways for degradation[37]. Lazar *et al*[38] reported that EDEM1 is upregulated to interact with HBV viral envelope proteins for degradation during HBV infection. Furthermore, the degradation of HBV envelope proteins was reversed by an autophagy inhibitor but not by a proteasomal inhibitor, implying that interacted envelope proteins are diminished by the autophagic degradation pathway. Furthermore, EDEM1-mediated degradation of HBV envelope proteins significantly suppressed the secretion of the enveloped and subviral HBV particles (Figure 2).

The relationship between autophagy and HBV in HBV-related tumorigenesis

Although many studies have reported that HBV induces autophagy, which promotes viral replication during HBV infection both *in vitro* and *in vivo*, the role of autophagy in HBV-associated tumorigenesis remains unclear. Many reports showed that autophagy plays a suppressive role in HCC tumorigenesis. The mice with mosaic deletion of *Atg5* or liver-specific *Atg7*$^{-/-}$ gene developed multiple liver tumors. In addition, autophagic gene *Beclin 1* expression was decreased in HCC tumors compared with adjacent non-tumor tissues[39,40]. However, Tian *et al*[41] claimed that autophagy shifts from a suppressive role to a promoter role in HCC development at the late stages by inhibiting the expression of various tumor suppressor genes including p53 and p21. In the following section, the current findings with respect to the mechanism by which autophagy influences HBV-related tumorigenesis and the implications for potential therapies are described.

The role of autophagy in HBV-related tumorigenesis: Both the HBV transgenic mouse model

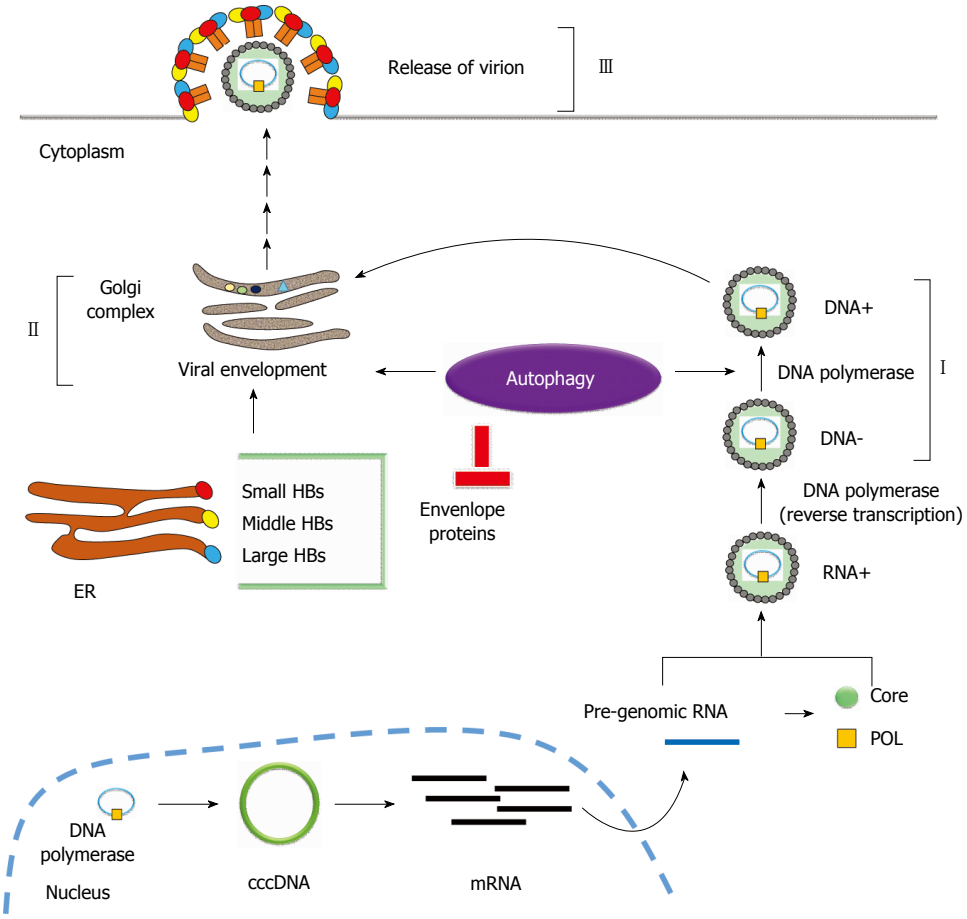

Figure 2 The relationship between hepatitis B virus-induced autophagy and hepatitis B virus replication. Hepatitis B virus (HBV)-induced autophagy regulates virus replication and maturation at different stages of HBV replication. (Ⅰ): Autophagy enhances HBV replication at the DNA replication stage; (Ⅱ): HBV-induced autophagy is required for viral envelopment. However, envelope proteins (HBs) could be eliminated *via* the autophagic degradation pathway (Ⅲ).

and human HCC specimens were analyzed to clarify the role of autophagy in HBV-related tumorigenesis. In contrast to autophagy induction by HBV infection, autophagy is suppressed in the liver tumors of a transgenic mouse model and HBV-related HCC. In the late stages of liver tumor development in the HBx transgenic mice model, autophagic activity is decreased following liver tumor formation. Both mRNA and protein levels of autophagy-related genes are reduced in the tumor tissues[42]. Qu *et al*[43] demonstrated that heterozygous deletion of *Beclin 1* in the HBV transgenic mice increased spontaneous malignancies and accelerated HBV-induced HCC. Furthermore, in a clinical investigation, Kotsafti *et al*[44] showed that the mRNA of *Beclin 1* was significantly lower in HCC tissues than in chronic hepatitis tissues. Consistent with these reports, we demonstrated that the protein levels of Atg5 and Beclin 1 were significantly lower in tumor tissues compared with the adjacent non-tumor tissues of HBV-associated HCC[43]. These results imply that autophagy plays a tumor suppressive role in hepatocarcinogenesis and is inhibited in HBV-associated tumorigenesis. However, the mechanism by which high autophagy during HBV infection shifts to low autophagy in HBV-related tumorigenesis remains unclear.

Autophagy-related potential therapy for HBV-related HCC: Although the role of autophagy in HBV-related tumorigenesis remains uncertain, several research groups have claimed that autophagy exerts a suppressive effect in HBV-related HCC tumorigenesis. Therefore, manipulating autophagic activity may have potential in the development of alternative therapies in the treatment of HCC. Different therapeutic strategies involving the regulation of autophagic activity in HCC treatment are described as: (1) Autophagy induces cell death; One method of suppressing HCC tumorigenesis is to induce hepatoma cell death. In contrast to type Ⅰ programmed cell death (apoptosis), the checkpoints of type Ⅱ programmed cell death autophagy are determined using several metabolic stresses[45]. Su *et al*[46] showed that soybean fermentation products containing live bacteria (SCB) were used to suppress liver tumor formation *via* induction of apoptosis and autophagy without significantly changing the mean body and liver weight in a syngeneic mouse model. Inhibition of autophagy by the inducer 3-MA suppresses SCB-induced apoptosis, indicating that SCB induced-autophagy

promotes apoptotic cell death. Furthermore, Zhang *et al*[47] designed an inhibitor (NTI-007) to treat HBV-infected cells, which targets the multiple transmembrane transporter as well as the functional receptor for HBV and leads to autophagic cell death of the HBV-infected cells. Taken together, the above findings indicate that some natural and synthetic compounds have chemotherapeutic potential against HBV infection through the induction of autophagic cell death; (2) Autophagy enhances antitumor immune responses: Another therapeutic approach in the treatment of HBV-related HCC involves stimulating antitumor immune responses[48,49]. HBx protein is an oncoprotein that has been detected in 80% of HBV-related HCC and is therefore a potential target for immunotherapy[50,51]. Yan *et al*[52] reported that significant antitumor immune responses could be induced by irradiation treatment of mice inoculated with the HBx gene expressing tumor cells. Irradiation of the tumor cells harboring the HBx gene induced the cytolytic T lymphocyte response which recognizes and lyses the HBx-expressing hepatoma cells. Accordingly, autophagosomes and autolysosomes were detected in the irradiated tumor cells expressing the HBx gene. These findings suggest that this autophagy-enhanced $CD8^+$ and $CD4^+$ T lymphocyte antitumor response could lead to the development of a promising therapy against HBx-related HCC; and (3) Autophagy degrades oncogenic miRNA: Selective autophagy has been reported to recognize and clean the specific cytosolic targets through autolysosome degradation[53]. Autophagy may suppress tumor formation of various cancers through selective degradation of the oncogenic molecules including proteins, microRNAs, and damaged organelles[54]. Lan *et al*[42] recently demonstrated that low autophagic activity together with high expression of miR-224 were detected both in HBx transgenic mice and HBV-related HCC patients. Autophagy selectively recruited the oncogenic miR-224, promoting tumor formation and cellular migration activity by silencing its target gene smad4. Furthermore, boosting autophagic activity by amiodarone, an autophagy inducer, suppressed oncogenic miR-224 expression and significantly reduced liver tumor formation in a rat orthotopic liver tumor model. These results demonstrated that besides proteins and organelles, microRNAs could also be degraded by autophagic degradation machinery to suppress HBV-related tumorigenesis.

Taken together, increased autophagic activity suppresses HBV-related HCC tumorigenesis through the induction of cell death, enhancement of the immune response, or by the degradation of oncogenic factors.

THE EFFECT OF MIRNAS ON HBV

MiRNAs regulate gene expression through post-transcriptional modification by targeting 3′ UTR of mRNA and silencing its translation[55]. During viral infection, the miRNAs either from infected viruses or the host cells could affect the target gene expression of viruses or host cells[56,57]. Here, we explored the mechanisms by which miRNAs regulate HBV replication as well as the underlying mechanism whereby miRNAs biogenesis is regulated by HBV.

MiRNAs affect HBV replication

HBV gene expression and replication can be regulated by host or viral miRNAs. These miRNAs may suppress or promote HBV infection at each stage of viral replication. We investigated the process by which miRNAs regulates HBV replication through direct targeting of mRNA in virus genes or by targeting host genes which are required for virus replication.

MiRNAs directly target HBV genes: Wu *et al*[58] used clinical HBV patient specimens and identified four human host miRNAs let-7, miR-345, miR-433 and miR-511 using target gene prediction software, which targeted the highly conserved HBV genes including the genes of polymerase, S and preC in different clades of HBV, indicating that these host miRNAs suppress HBV replication. Moreover, Kohno *et al*[59] used SCID mice harboring humanized hepatocyte cells to identify miRNAs upregulated by HBV infection in the liver tissue. MiR-1231 was the most upregulated miRNA which suppressed HBV replication through the inhibition of HBV core protein expression. In addition, miR-125a-5p, miR-199a-3p and miR-210 have also been reported to be able to suppress HBV replication by targeting and reducing S gene expression[60,61]. In addition, one predicted HBV miRNA located in the precore region (CAUGUCCUACUGUUCAAGCCUC) may target three HBV genes (large S, polymerase and X genes) and serves as the feedback regulation of HBV replication to maintain the latent status[62]. Taken together, miRNAs may suppress HBV replication through the suppression of diverse viral gene expression.

MiRNAs regulate HBV replication by targeting host genes: It has been shown that HBV-synthesized miRNAs may target its own genes to affect its replication, but host miRNAs could also affect virus replication by targeting host genes. Guo *et al*[63] reported that miR-372/373 was upregulated in HepG2.2.15 cells, stably transfected with a complete HBV genome, and miR-372/373 enhanced the expression levels of HBV core-associated DNA and HBx protein. They found that miR-372/373 silenced the host transcription factor nuclear factor I/B (NFIB), which is a repressor bound to the enhancer 1 region of HBV genome including the S gene promoter, suggesting that HBV enhances its replication through the upregulation of miR-372/373 to suppress NFIB

expression. In addition, Jin *et al*[64] showed that miR-501 is overexpressed in HBV-infected cell lines and in HCC specimens, thereby promoting HBV replication by suppressing expression of HBXIP, which is a host protein interacting with HBx protein at its transactivation domain to repress its function. These findings imply that HBV enhances its replication by regulating host miRNAs expression to suppress anti-HBV proteins from the host cell.

One research group identified potential miRNAs which regulate HBV replication by screening a library of miRNAs. Zhang *et al*[65] reported that overexpression of miR-1 in hepatoma cells increased HBV replication by silencing histone deacetylase 4 (HDAC4) and increased the expression of farnesoid X receptor (FXRA). FXRA, a transcriptional factor, interacts with RXRA (FXRA/RXRA) and then binds to the HBV core promoter to enhance its activity. HDACs regulate acetylation of H3/H4 histones bound to the cccDNA of HBV and this epigenetic modification regulates the transcription and replication of HBV. In contrast, Hu *et al*[66] reported that miR-141 identified by screening 64 miRNAs suppressed HBV replication by silencing host transcription factor PPARA, which is an essential factor for HBV pregenomic RNA synthesis and viral replication. These findings summarize the miRNAs which affect virus replication through the suppression of host genes.

MiR-15b promotes HBV replication by silencing hepatocyte nuclear factor 1 alpha, a repressor of the HBV enhancer Ⅰ region. HBV replication upregulates HBx protein expression, which then suppresses miR-15b expression[67]. The relationship between HBV and microRNA suggests a feedback regulation between HBV replication and host miRNAs expression. MicroRNA may play dual roles in HBV replication. MiR-122 is highly expressed in the liver of HCC patients and is positively associated with HBV infection. Qiu *et al*[68] showed that miR-122 promotes HBV replication by silencing heme oxygenase-1 which interacts and reduces the stability of the HBV core protein. The above findings are in contrast to the results of a study by Ji *et al*[69] that showed miR-122 suppresses HBV replication.

HBV regulates the biogenesis of miRNAs

HBV may affect microRNA biogenesis to alter the expression of miRNAs. MiRNAs are initially transcribed by RNA polymerase Ⅱ and Ⅲ to form primary miRNA (pri-miRNA, 300-1000 nucleotides) in the nucleus, which is subsequently processed by the microprocessor consisting of RNase, Drosha, and DGCR8 (DiGeorge syndrome critical region gene 8) resulting in a shorter precursor miRNA (pre-miRNA, 70-90 nucleotides). Pre-miRNA is then exported by the nuclear export receptor exportin-5 and RAN-GTP to the cytoplasm, where it is processed by Dicer to generate the mature miRNA. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) and then regulates gene silencing. HBV affects the biogenesis of miRNAs at various stages through different mechanisms.

HBV affects miRNA transcription and stability: MiR-122 is a liver-specific miRNA which maintains liver function. Dysregulated miR-122 affects virus replication of diverse hepatitis viruses[70]. In HCV infection, miR-122 promotes its replication by interacting with the 5'-non-coding region (5'-NCR) of HCV[71]. In contrast, miR-122 inhibits HBV replication and is significantly downregulated during HBV infection[72]. HBV suppresses miR-122 using various mechanisms. Song *et al*[73] reported that HBV suppresses miR-122 expression *via* the HBx protein, which interacts with peroxisome proliferator activated receptor-gamma (PPARγ) and suppresses the transactivation function of PPARγ/RXRα complex on the promoter of miR-122 (Figure 3A). In addition, HBV downregulates miR-122 expression by influencing miRNA stabilization. Peng *et al*[74,75] demonstrated that HBx suppresses transcription of Gld2 protein (germline development 2) which specifically increases miR-122 stabilization by catalyzing 3' monoadenylation and reduces miR-122 maturation (Figure 3B). Li *et al*[76] showed that HBV suppresses miR-122 expression and causes the upregulation of the miR-122 target gene PTTG (pituitary tumor-transforming gene 1 binding factor), which leads to the promotion of HCC tumorigenesis. They further identified the miR-122 complementary targeting site on the four mRNAs of HBV, including pre-C/C, pre-S, S and X gene. Therefore, HBV mRNA functions as a sponge to bind and reduce miR-122 expression (Figure 3C). Taken together, the above findings provide mechanistic insights into the differential regulation of miRNAs by HBV.

HBV affects RNase expression: HBV infection alters miRNA expression profiles in infected cell lines and clinical HCC patients[69,77]. HBV could also regulate miRNA biogenesis through miRNA-related RNases. Drosha and Dicer are two miRNA processing components required for miRNA maturation. Recent evidence showed that HBV dysregulates miRNA biogenesis by affecting these two proteins. Ren *et al*[78] reported that expression of both Drosha mRNA and protein was suppressed in cells expressing the HBV genome. They revealed that HBx protein downregulates Drosha expression through the reduction of its promoter activity (Figure 4). Furthermore, HBV upregulates the expression of the repressor YY1 to suppress Drosha cofactor DGCR8 expression and subsequently represses DGCR8 promoter activity (Figure 4)[79]. Lund *et al*[80] identified three single nucleotide polymorphisms (SNPs) including DICER rs1057035 and RAN rs3803012 in HBV-related clinical HCC specimens, and these SNPs were found to be associated with the risk of HBV-related HCC

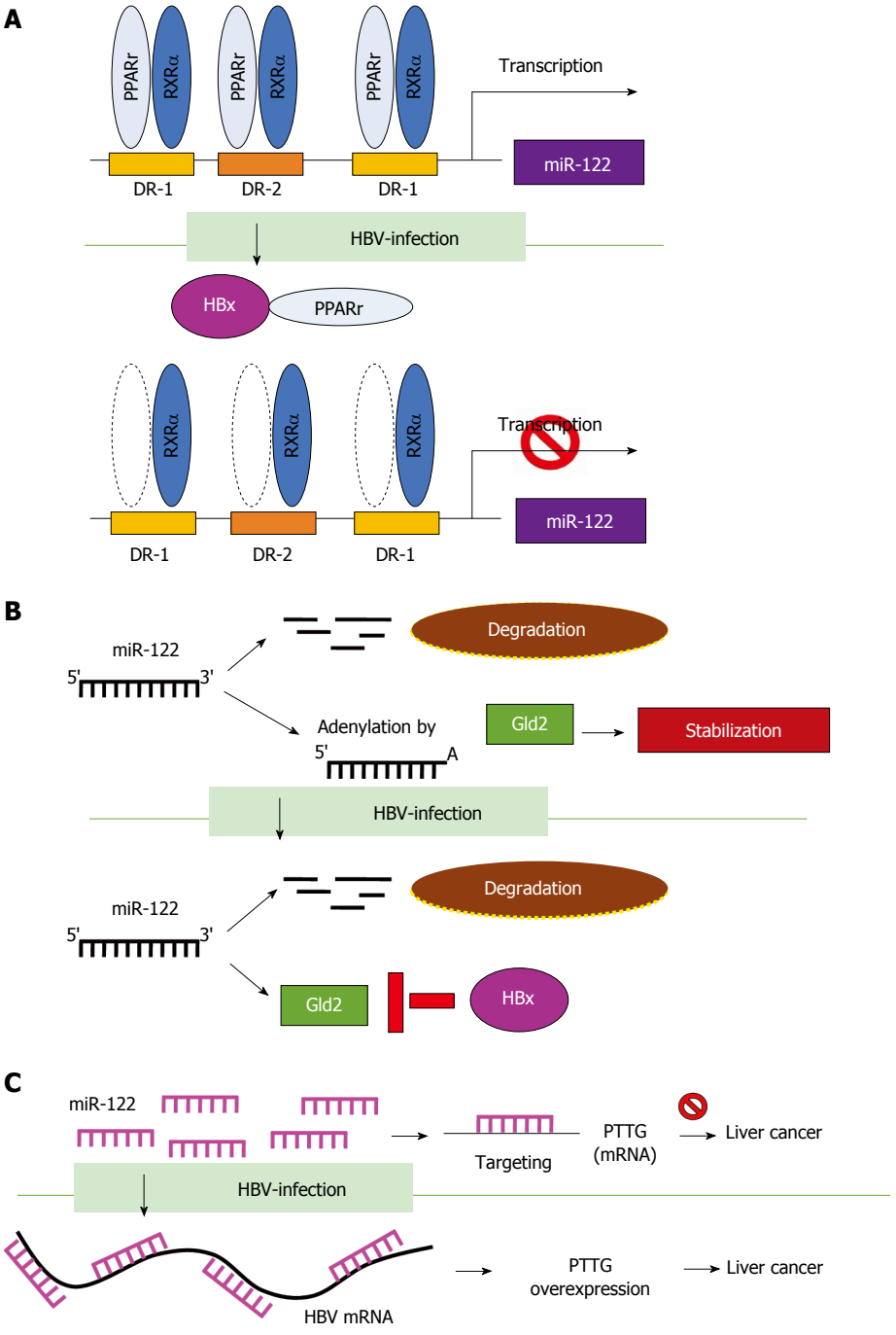

Figure 3 Hepatitis B virus affects microRNA transcription and stability. Hepatitis B virus (HBV) suppresses miR-122 through different mechanisms. A: HBV suppresses miR-122 promoter activity and expression through transcriptional regulation by directly interacting with peroxisome proliferator activated receptor-gamma (PPARγ); B: HBx suppresses transcription of Gld2 to decrease miR-122 stability; and C: HBV functions as a sponge to bind miR-122 and reduces its expression. RXRa: Retinoid X Receptor, Alpha; Gld2: Germline development 2.

occurrence. They found that the SNPs located on the 3'UTR of DICER and RAN genes affect the binding affinity of miR-574-3p and miR-199a-3p, respectively. Dysregulated DICER and RAN affect miRNA biogenesis in HBV-related diseases (Figure 4)[81]. Taken together, these findings show that HBV suppresses miRNA biogenesis through the repression of the promoter activity of mature miRNA-related genes.

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, we provide a detailed description of our current understanding of the relationships among HBV, autophagy, and miRNAs. However, the following questions remain unresolved: (1) Based on manipulation of autophagic activity, therapeutic

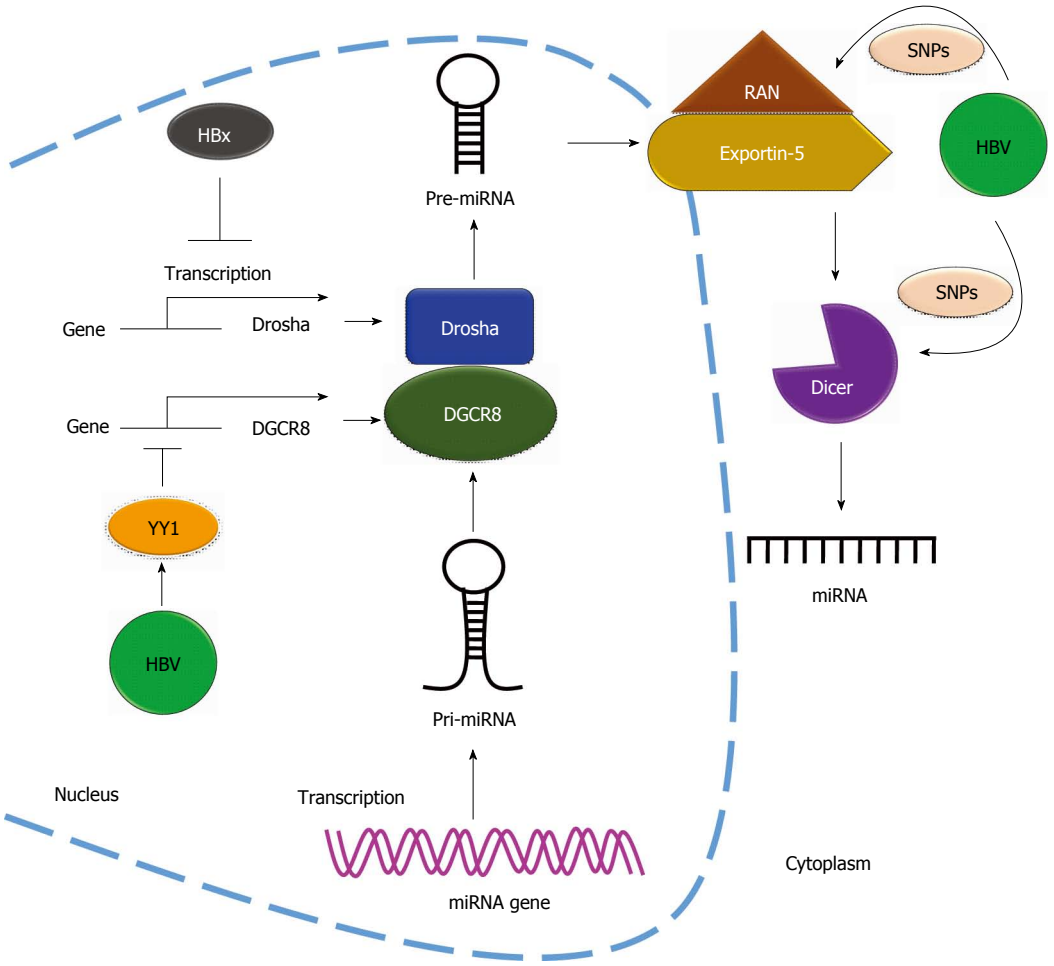

Figure 4 Hepatitis B virus regulates microRNAs biogenesis. Hepatitis B virus (HBV) regulates the biogenesis of miRNA by affecting the miRNA-related Rnases. HBx protein downregulates Drosha expression through reduction of the transcriptional activity of Drosha promoter and upregulates the expression of transcription factor YY1 to suppress DGCR8 expression by repressing DGCR8 promoter activity. Single nucleotide polymorphisms (SNPs) located on 3'UTR of Dicer and RAN genes affect the biogenesis of miRNA in HBV-related diseases. DGCR8: DiGeorge syndrome critical region gene 8.

strategies against the diseases of HBV-acute infection and HBV-related HCC tumorigenesis should be different. Because autophagy plays a promoting role in HBV replication but a suppressive role in HBV-associated tumorigenesis, the timing of HBV infection as well as the tumorigenesis status of the HCC patient should be carefully evaluated before considering an autophagy-related therapy. Further studies on combination therapy using autophagy inducers and anti-viral drugs for HBV-related diseases should be considered; (2) The role of secretory autophagy in HBV infection and its related pathogenesis could be further explored. Secretory autophagy has been reported to regulate cytokines secretion including IL-1β, IL-18 and HMGB1 (high-mobility group box 1 protein). Interestingly, IL-1β and HMGB1 were significantly upregulated in the serum of patients with hepatitis B virus-related acute-on-chronic liver failure[82,83], and the expression of IL-18 in peripheral blood mononuclear cells from patients with acute HBV infection was higher compared with the expression in patients with chronic HBV infection[84]. These reports imply that HBV may affect release of these cytokines through HBV-induced secretory autophagy. Furthermore, the HBV gene in liver-specific *Atg5* knockout transgenic mice showed significantly lower expression of HBsAg and HBeAg in the serum compared with the wild-type mice[33]. It is possible that HBV-induced secretory autophagy contributes to release of viral antigens. Further studies are needed to clarify the role of secretory autophagy in HBV infection and pathogenesis; (3) The role of HBV-affected miRNA biogenesis in HBV replication remains undetermined. Current evidence indicates that HBV suppresses miRNAs biogenesis by affecting RNases and cofactors of miRNA biogenesis, but whether this is beneficial or harmful for HBV replication remains unclear; and (4) The effect of autophagy and/or miRNAs on HBV recurrence and latency has not been clearly elucidated. During HBV infection, virus infection is defined as acute in the initial period of several months and is defined as chronic when the infection period has lasted for many years. HBV is actively replicated during the acute infection phase followed by recovery in the presence of the host immune responses. However, during the chronic infection, the immune-tolerant phase is characterized by a high

level of HBV DNA. When host immunity shifts to the immune-active status, HBV accordingly becomes less replicative. However, HBV may be reactivated to a high replication status in certain HBV carriers. These phenomena imply that HBV continuously undergoes reactivation and latency. Further exploration is needed to clarify the mechanisms by which autophagy and/or miRNAs affect HBV recurrence and latency in HBV-infected patients.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Naturally derived anti-hepatitis B virus agents and their mechanism of action

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Abstract

Despite that some approved drugs and genetically engineered vaccines against hepatitis B virus (HBV) are available for HBV patients, HBV infection is still a severe public health problem in the world. All the approved therapeutic drugs (including interferon-alpha and nucleoside analogues) have their limitations. No drugs or therapeutic methods can cure hepatitis B so far. Therefore, it is urgently needed to discover and develop new anti-HBV drugs, especially non-nucleoside agents. Naturally originated compounds with enormous molecular complexity and diversity offer a great opportunity to find novel anti-HBV lead compounds with specific antiviral mechanisms. In this review, the natural products against HBV are discussed according to their chemical classes such as terpenes, lignans, phenolic acids, polyphenols, lactones, alkaloids and flavonoids. Furthermore, novel mode of action or new targets of some representative anti-HBV natural products are also discussed. The aim of this review is to report new discoveries and updates pertaining to anti-HBV natural products in the last 20 years, especially novel skeletons and mode of action. Although many natural products with various skeletons have been reported to exhibit potent anti-HBV effects to date, scarcely any of them are found in the list of conventional anti-HBV drugs worldwide. Additionally, in anti-HBV mechanism of action, only a few references reported new targets or novel mode of action of anti-HBV natural products.

Key words: Natural product; Hepatitis B virus; Structure; Mechanism of action; Drug target

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Core tip: Hepatitis B virus (HBV) infection continues to be a significant health problem in the world. The urgent need for new anti-HBV drugs is a global concern.

Naturally originated compounds with various skeletons and diverse biological activities provide a large reservoir for finding novel anti-HBV leads or candidates. In this review, the anti-HBV natural products are classified according to their structure types such as terpenes, lignans, phenolic acids, polyphenols, lactones, alkaloids and flavonoids. Furthermore, novel mode of action or new targets of some representative anti-HBV natural products are also discussed.

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INTRODUCTION

Hepatitis B is an infectious disease caused by hepatitis B virus (HBV), which infects the liver of Hominoidea, including humans. The infection of hepatocytes by HBV causes an inflammatory response and the subsequence damage of hepatocytes. Although there is an effective anti-HBV vaccine, chronic infection poses still a huge health burden all over the world[1]. In some hyperendemic areas (such as sub-Saharan Africa and East Asia), the prevalence of hepatitis B is over 8%[2]. Moreover, the people infected with HBV are nearly 3.7% of the global population (more than 240 million people). Of these, the annual number of deaths from HBV is estimated 786000[3]. Current USFDA approved therapies for HBV infections including immune modulators (interferon-alpha and pegylated interferon-alpha) and nucleoside analogues (lamivudine, entercavir, adefovir dipivoxil, telbivudine and tenofovir) have also been approved for the treatment of chronic HBV infection. However, the above-mentioned anti-HBV drugs have their limitations. For example, interferon-alpha has limited efficacy and high incidence of adverse reactions; whereas nucleoside analogues are very efficient HBV DNA inhibitors, but long-term use of these agents may result in drug resistance in a significant number of patients and does not result in the clearance of HBV genome from the infected liver[4]. Therefore, there is an obvious and urgent need to develop novel and efficient anti-HBV agents.

Natural products are important sources of new drugs and leads besides tailored synthesis[5]. To meet the pressing need, the development of naturally derived antiviral agents may be a promising approach[6]. In this review, anti-HBV natural products are reviewed and discussed according to their chemical classes. Attempts are made to cover as many reports as possible on compounds of natural origin that inhibit HBV replication giving special emphasis to new discoveries including novel structures and targets.

TERPENOIDS

Terpenoids are the largest class of natural products basd on a various but definite number of five-carbon isoprene units and play a variety of roles in mediating antagonistic interactions especially in traditional herbal remedies. Many natural terpenes possess promising anti-HBV activity. Betulinic acid was isolated from *Pulsatilla chinensis* and exhibited an inhibitory effect on HBV replication by down-regulating expression of manganese superoxide dismutase (SOD2) in HBV transgenic mice, which was followed by mitochondrial reactive oxygen species (ROS) generation and dysfunction[7]. Further investigation showed that SOD2 expression was repressed by betulinic acid-induced dephosphorylation of cAMP-response element-binding protein (CREB) at Ser133, a critical transcription factor for SOD2 transcription. The inhibition of HBV replication by betulinic acid was blocked by SOD2 overexpression, and that SOD2 knockdown validated this effect, indicating that the anti-HBV effect of betulinic acid may be achieved by modulating the balance of mitochondrial redox. Betulinic acid suppressed SOD2 expression, with enhanced ROS generation in the liver followed by the significant decline of HBV replication in HBV transgenic mice, suggesting that betulinic acid could be a good anti-HBV candidate[7].

Asiaticoside from *Hydrocotyle sibthorpioides* inhibited effectively the HBV surface antigen (HBsAg), HBV e antigen (HBeAg), HBV DNA and covalently closed circular DNA (cccDNA) levels in a dose dependent manner[8]. Moreover, asiaticoside significantly decreased the transcription and replication of viral DNA by suppressing the core, S1, S2, and X gene promoter activities. Additionly, asiaticoside reduced markedly duck hepatitis B virus (DHBV) replication. On the third day after drug withdrawal, the DHBV DNA, DHBsAg and DHBeAg levels were enhanced in serum, but the rebound levels in the asiaticoside-treated groups are milder compared with the 3TC-treated group. Furthermore, asiaticoside could alleviate liver damage[8]. Astataricusones B and epishionol were obtained from the roots and rhizomes of *Aster tataricus L. f.* Astataricusones B showed an inhibitory effect on HBsAg secretion with a 50% inhibition concentration (IC_{50}) value of 23.5 μmol/L, while astataricusones B and epishionol exhibited inhibitory effects on HBeAg secretion with IC_{50} values of 18.6 and 40.5 μmol/L, respectively. Astataricusones B and epishionol also exhibited inhibitory activities on HBV DNA replication with IC_{50} values of 2.7 and 30.7 μmol/L, respectively[9]. Pumilaside A from *Artemisia capillaris* could inhibit not only the secretion of HBsAg and HBeAg in HepG 2.2.15 cell line, but also HBV DNA replication with IC_{50} values of 15.02 μmol/L (SI = 111.3), 9.00 μmol/L (SI = 185.9) and 12.01 μmol/L (SI = 139.2), respectively[10,11]. Dehydroandrographolide and andrographolide from *Andrographis paniculata* possessed activity against

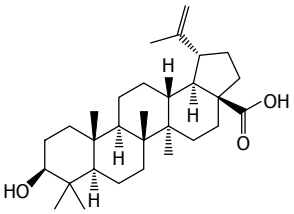
HBV DNA replication with IC_{50} values of 22.58 and 54.07 μmol/L and low SI values of 8.7 and 3.7 μmol/L, respectively[12]. Sweriyunnangenin A and 3-epitaraxerol from *Swertia yunnanensis* showed activities against the secretion of HBsAg (IC_{50} values of 0.28 and 0.70 mmol/L) and HBeAg (IC_{50} values of 0.29 and 1.41 mmol/L), respectively[13]. Alisol A from the rhizomes of *Alisma orientalis* exhibited potent activities against HBsAg (IC_{50} = 0.039 mM) and HBeAg (IC_{50} > 0.028 mmol/L) secretion with remarkable selective indices (SIHBsAg = 1.6, SIHBeAg < 0.03), respectively[14].

Alisol A 24-acetate, 25-anhydro alisol A, 13β,17β-epoxy alisol A, alisol B 23-acetate, alisol F, and alisol F 24-acetate from the rhizomes of *Alisma orientalis* exhibited an inhibitory activity on HBsAg secretion of HepG2.2.15 cells with IC_{50} values of 2.3, 11.0, 15.4, 14.3, 0.6 and 7.7 mmol/L, and on HBeAg secretion with IC_{50} values of 498.1, 17.6, 41.0, 19.9, 8.5 and 5.1 mmol/L, respectively[15,16]. Hemslecin A from the genus *Hemsleya* showed an effect against HBV DNA replication (IC_{50} = 11.2 lM, SI = 5.8) in HepG2.2.15 cells[17]. Perovskatone A and demethylsalvicanol from *Perovskia atriplicifolia* possessed a superior inhibitory effect on HBsAg (IC_{50}: 0.16 mmol/L and 2.23 mmol/L) and HBeAg (IC_{50}: 1.54 mmol/L and 3.67 mmol/L), respectively[18]. Methyl helicterate (MH) from the Chinese herb *Helicteres angustifolia* significantly decreased the HBsAg/HBeAg secretion, the HBV DNA/cccDNA levels, and the amount of viral RNA in HepG2.2.15 cells; in DHBV-infected ducklings, MH significantly reduced the serum DHBV DNA, liver total viral DNA, and cccDNA levels, and improved the liver pathological changes[19]. Caudatin from *Cynanchum auriculatum* had an inhibitory activity against HBsAg secretion and HBV DNA replication with the IC_{50} values of 142.67 μmol/L (SI = 1.7), 40.62 mmol/L (SI = 6.0), respectively, and 3-*O*- (3, 4, 5-trimethoxy) cinnamoyl caudatin had the novel mechanism of anti-HBV action by interfering HBV enhancers and promoters[20]. Phyllanthacidoid acid methyl ester and phyllanthacidoids A, B, C, D, F, G, H, I and M from *Phyllanthus acidus* Skeels displayed potential anti-HBV activities, with IC_{50} values of 0.8-36 μmol/L against HBsAg and HBeAg, and the results indicated that the 5-ketal group and sugar moieties had contributions to the selectivity of HBsAg and HBeAg[21]. Phyllaemblicins G6-G8 and phyllaemblicin F from *Phyllanthus emblica* displayed potential anti-HBV activities, especially for phyllaemblicin G6 with IC_{50} values of 8.53 ± 0.97 and 5.68 ± 1.75 μmol/L towards the HBsAg and HBeAg secretion, respectively[22]. Ursolic acid from the heartwood of Streblus asper showed an anti-HBV effect, with IC_{50} values of 89.91 μmol/L for HBsAg and IC_{50} values of 97.61 μmol/L for HBeAg, respectively[23]. Oleanolic acid from Swertia yunnanensis inhibited the secretion of HBsAg with an IC_{50} value of 1.26 mmol/L and HBeAg with an IC_{50} value of 0.94 mM, respectively[13]. Glycyrrhizin and its metabolite glycyrrhetinic acid (GA) are the main effective constituents of *Licorice* root (*Glycyrrhizae glabra*) for the treatment of chronic hepatitis B. GA exhibited inhibitory activities against HBV DNA replication (IC_{50} = 39.28 μmol/L), HBV surface antigen secretion (IC_{50} = 20.86 μmol/L) and HBV e antigen secretion (IC_{50} = 10.49 μmol/L)[24] (Figure 1).

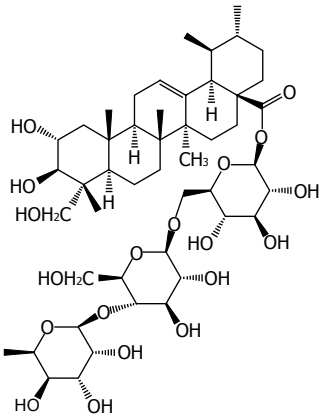
LIGNANS

Lignans are a widely distributed phenylpropanoid derivatives in plants and are classified into five main structure types including lignans, oligomeric lignans, hybrid lignans, norlignans and neolignans. Lignan is also an important natural anti-HBV conponent. Helioxanthin (HE-145) was isolated from the heartwood of *Taiwania cryptomerioides* Hayata, and it was found to inhibit the gene expression and replication of HBV in HCC cells[25]. To elucidate the mode of anti-HBV action of HE-145, the authors examined the effects of HE-145 on HBV promoter activities by the luciferase reporter assay. The rusults showed that HE-145 inhibited selectively core promoter (CP) and surface antigen promoter II (SPII), but had no effect on X gene promoter (Xp) and surface antigen promoter I (SPI). Meanwhile, the inhibition of HE-145 on CP or SPII promoter was not observed in non-liver cells such as 293T and HeLa, suggesting that the inhibitory effects of HE-145 on SPII or CP could be liver-specific. The electrophoretic mobility shift assay indicated that HE-145 reduced the DNA-binding activity of HepA2 cell nuclear extract to several cis elements on CP, including alpha-fetoprotein transcription factor binding site, peroxisome proliferator-activated receptor (PPAR) binding site, and Sp1 binding site. Anomalous expression of PPAR-γ or hepatocyte nuclear factor 4-α reversed partially the HE-145-mediated HBV RNA suppression[25]. In short, HE-145 possess a unique anti-HBV mechanism and could be a novel class of potential anti-HBV agents. Nevertheless, what are its specific mechanisms of action? Down-regulation of these transcription factors by HE-145 or inactivation of a common coactivator? Hence, further studies are required to answer these questions.

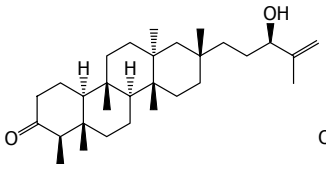
A lignan glycoside (+)-cycloolivil-4′-*O*-β-*D*-glucopyranoside from *Swertia chirayita* exhibited an inhibitory effect not only on the HBsAg and HBeAg secretion with IC_{50} values of 0.31 ± 0.045 mmol/L (SI = 4.29) and 0.77 ± 0.076 mmol/L (SI = 1.75), respectively, but also on the replication of HBV DNA with an IC_{50} value of 0.29 ± 0.034 mmol/L (SI = 4.66)[26,27]. Schisanwilsonin D, schisantherin C, deoxyschizandrin and (+)-gomisin K3 were isolated from the fruits of *Schisandra wilsoniana* and showed anti-HBV effects; schisantherin C exhibited the strongest anti-HBV effect by diminishing HBsAg and HBeAg secretion by 59.7% and 34.7%, respectively[28]. Honokiol from *Streblus asper* showed marked anti-HBV activities with IC_{50} values of 3.14 μmol/L (SI = 21.47)



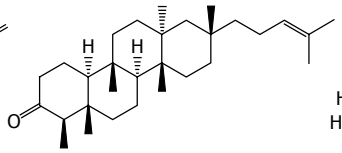
Betulinic acid



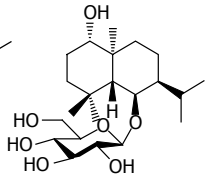
Asiaticoside



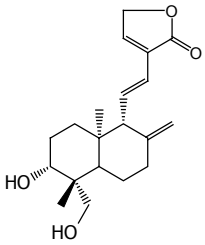
Astataricusones B



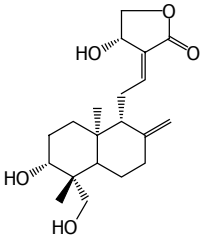
Epishionol



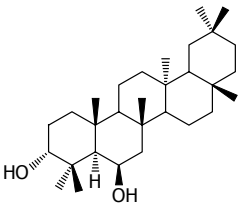
Pumilaside A



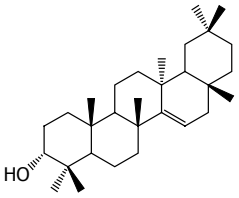
Dehydroandrographolide



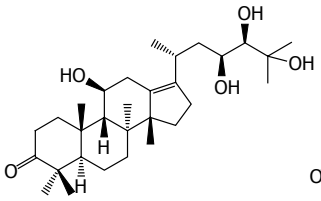
Andrographolide



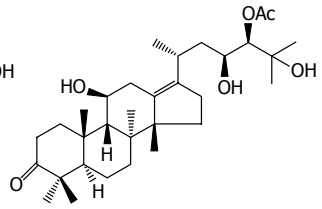
Sweriyunnangenin A



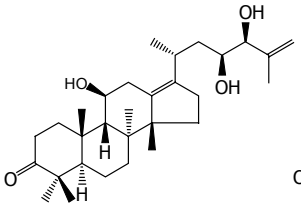
3-Epitaraxerol



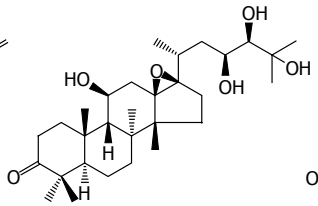
Alisol A



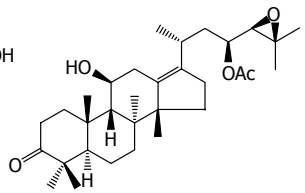
Alisol A 24-acetate



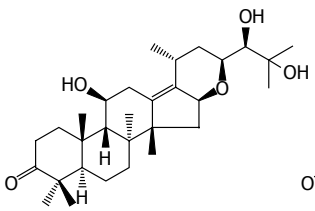
25-Anhydro alisol A



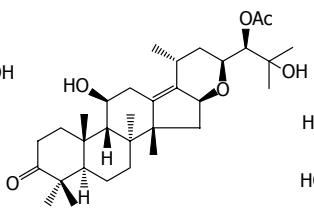
13β,17β-Epoxy alisol A



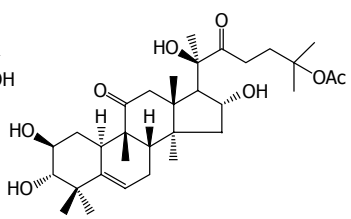
Alisol B 23-acetate



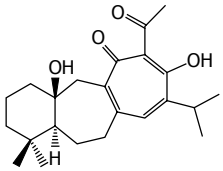
Alisol F



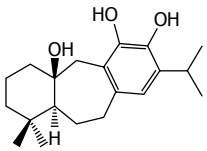
Alisol F 24-acetate



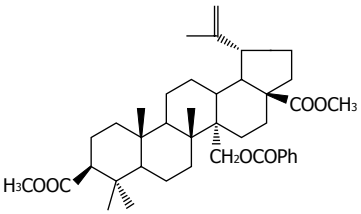
Hemslecin A



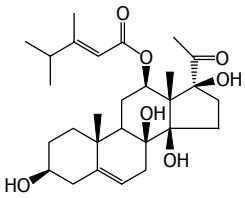
Perovskatone A



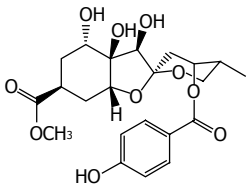
Demethylsalvicanol



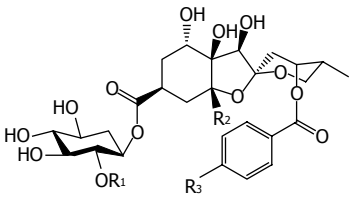
Methyl helicterate

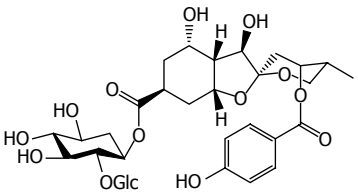


Caudatin



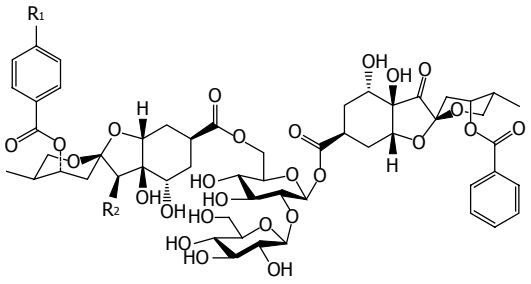
Phyllanthacidoid acid methyl ester



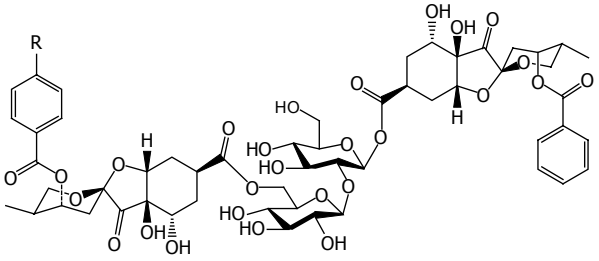


Phyllanthacidoid A: R1 = N-Ac-GlcN; R2 = H; R3 = OH
Phyllanthacidoid B: R1 = N-Ac-GlcN; R2 = H; R3 = H
Phyllanthacidoid C: R1 = Glc; R2 = H; R3 = H
Phyllanthacidoid D: R1 = Glc; R2 = H; R3 = OH
Phyllanthacidoid F: R1 = N-Ac-GlcN; R2 = OH; R3 = OH
Phyllanthacidoid G: R1 = Glc; R2 = OH; R3 = OH
Phyllanthacidoid H: R1 = Glc(2-1)Glc; R2 = OH; R3 = OH
Phyllanthacidoid I: R1 = Glc; R2 = OCH_3; R3 = OH

Phyllanthacidoid M



Phyllaemblicin G6: R1 = H; R2 = OH
Phyllaemblicin G7: R1 = OH; R2 = O=



Phyllaemblicin G8: R = OH
Phyllaemblicin F: R = H

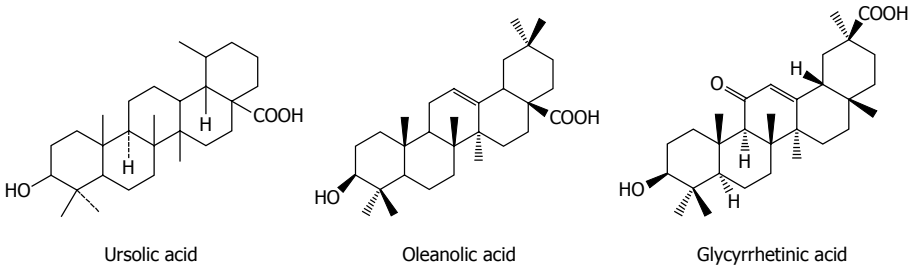


Figure 1 Chemical structures of representative anti-hepatitis B virus natural terpenes.

and 4.74 μmol/L (SI = 14.22) for HBsAg and HbeAg, respectively[29]. Four lignans from the root of *Streblus asper*, (7′R,8′S,7″R,8″S)-erythro-strebluslignanol G, magnolol, isomagnolol and isolariciresinol, exhibited significant anti-HBV effects in HepG2.2.15 cells with IC_{50} values of 1.58, 2.03, 10.34 and 3.67 μmol/L, respectively, for HBsAg, and of 3.24, 3.76, 8.83 and 14.67 μmol/L, respectively, for HBeAg. (7′R,8′S,7″R,8″S)-erythro-strebluslignanol G and magnolol showed significant anti-HBV activities to inhibit the replication of HBV DNA with IC_{50} values of 9.02 and 8.67 μmol/L, respectively[30]. 9-β-xylopyranosyl-isolariciresinol from the stem bark of *Streblus asper* exhibited a significant anti-HBV activity in Hep G2.2.15 cells with an IC_{50} value of 6.58 μmol/L for secretion of HBsAg, and of 24.86 μmol/L for secretion of HBeAg[31]. Niranthin and nirtetralin B are two new lignans from *Phyllanthus niruri L*. (Euphorbiaceae). Niranthin and nirtetralin B inhibited HBV antigen secretion in HepG2.2.15 cells with IC_{50} values for HBsAg of 15.6 and 17.4 μmol/L, IC_{50} values for HBeAg of 25.1 and 63.9 μmol/L, respectively. In ducklings with DHBV, niranthin and nirtetralin B significantly decreased the serum HBsAg, HBeAg, DHBV DNA, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, and improved the pathological changes of liver tissue, respectively[32,33]. Three lignans, (+)-(7′*S*, 7″*S*, 8′*R*, 8″*R*)-4, 4′, 4″-trihydroxy-3, 5′, 3″-trimethoxy-7-oxo-8-ene [8-3′, 7′-*O*-9″, 8′-8″, 9′-*O*-7″] lignoid, (1*S*)-4-hydroxy-3-[2-(4-hydroxy-3-methoxy-phenyl)-1-hydroxymethyl-2-oxo-ethyl] -5-methoxy-benzaldehyde and herpetetrone from the seeds of *Herpetospermum caudigerum* Wall displayed inhibitory effects on HBsAg secretion with IC_{50} values of 20.5, 0.34, and 4.89 μmol/L, and on HBeAg secretion with IC_{50} values of 3.54, 0.048, and 8.02 μmol/L, respectively[34] (Figure 2).

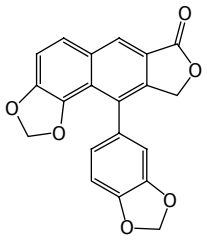
PHENOLIC ACIDS

Phenolic acids are the different types of naturally occurring aromatic compounds containing a phenolic ring and a carboxylic acid function. Interestingly, some phenolic acids often have antiviral effects. 3,4-*O*-dicaffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid isolated from *Laggera alata* significantly suppressed the HBsAg and HBeAg production and their inhibitory rates on the HBsAg and HBeAg expression were 89.96%/86.9% and 81.01%/72.9%, respectively. Meanwhile, 3,4-*O*-dicaffeoylquinic acid markedly decreased the HBV cccDNA content and significantly increased the heme oxygenase-1 (HO-1) expression in HepG2.2.15 cells and HBV transgenic mice. 3,5-*O*-dicaffeoylquinic acid showed a similar effect. Because HO-1 can destabilize the HBV core protein, this observation suggests that HO-1 overexpression may contribute to the antiviral activity of the two compounds by decreasing the stability of the HBV core protein, which blocks the refill of nuclear HBV cccDNA[35,36]. Seven quinic acid derivatives, 3,4-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoyl-muco-quinic acid, 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-(*E*)-*p*-coumaroylquinic acid, isolated from the aerial parts of *Lactuca indica L.* (Compositae), effectively reduced HBV DNA level in HepG2.2.15 cells, and treatment with 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoyl-muco-quinic acid and 5-*O*-(*E*)-*p*-coumaroylquinic acid led to a marked decline in the extracellular HBV DNA level[37].

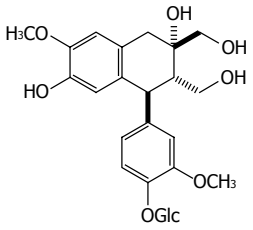
Chlorogenic acid and its related compounds are widely distributed in the leaves and fruits of dicotyledonous plants such as coffee beans and have a diverse antiviral activity. Chlorogenic acid, quinic acid and caffeic acid inhibited HBsAg production as well as HBV-DNA replication in HepG2.2.15 cells. Chlorogenic acid and caffeic acid also decreased the serum DHBV level in a DHBV-infected duckling model[38] (Figure 3).

POLYPHENOLS

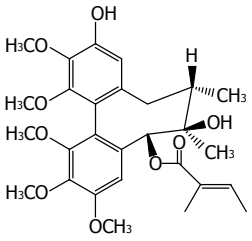
Polyphenols are some secondary metabolites containing two or more phenolic rings and are widely involved in defense against aggression by pathogens such as virus and bacteria. The compound LPRP-Et-97543 from *Liriope platyphylla* roots was observed to have potential anti-HBV effects in HepG2.2.15 cells. The antiviral mode of LPRP-Et-97543 was further studied using the HBV-transfected Huh7 cells. The effects of LPRP-Et-97543 on the viral precore/pregenomic and S/preS RNA were apparent during HBV gene expression. Analysis of promoter activity indicated that LPRP-Et-97543 markedly decreased S, preS, and Core but not X promoter activities. Further studies showed that



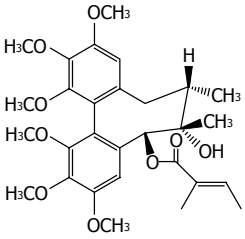
Helioxanthin (HE-145)



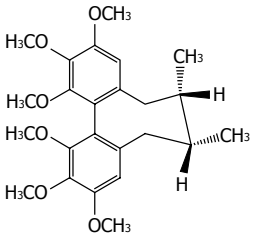
(+)-Cycloolivil-4′-*O*-*β*-*D*-glucopyranoside



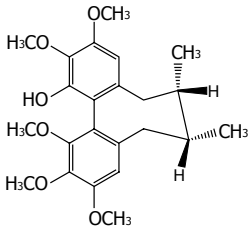
Schisanwilsonin D



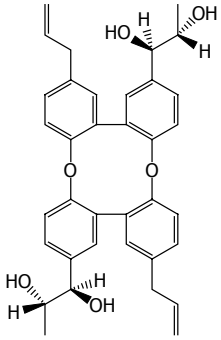
Schisantherin C



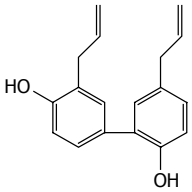
Deoxyschizandrin



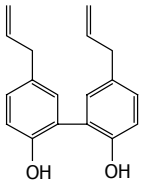
(+)-gomisin K3



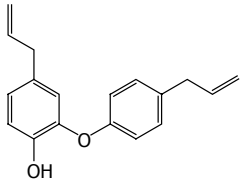
(7'R, 8'S,7"R,8"S)-erythro-strebluslignanol G



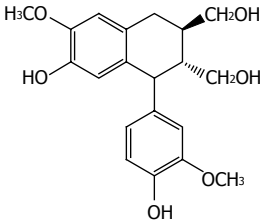
Honokiol



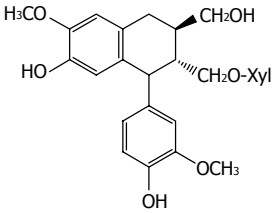
Magnolol



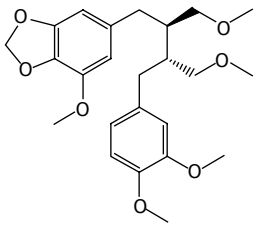
Isomagnolol



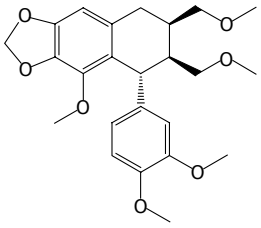
Isolariciresinol



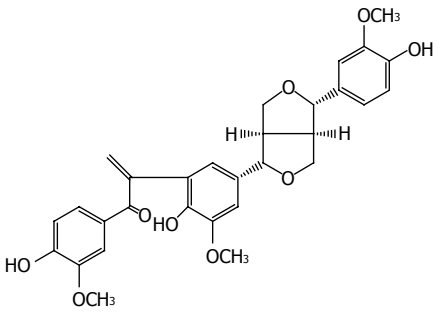
9-β-Xylopyranosyl-isolariciresinol



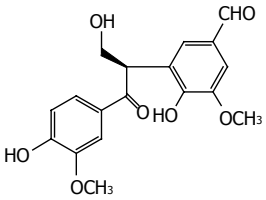
Nirtetralin



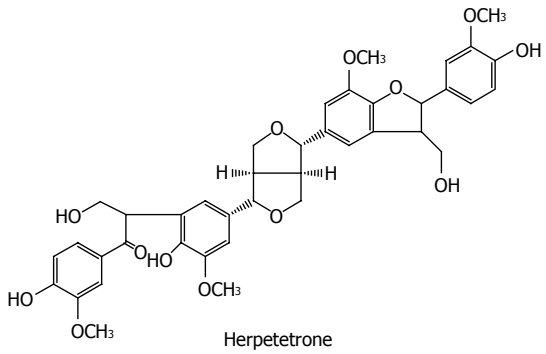
Nirtetralin B



4,4′,4″-Trihydroxy-3, 5′, 3″-trimethoxy-7-oxo-8-ene [8-3′,7′-*O*-9″,8′-8″,9′-*O*-7″] lignoid



(1*S*)-4-hydroxy-3-[2-(4-hydroxy-3-methoxy-phenyl)-1-hydroxymethyl-2-oxo-ethyl]-5-methoxy-benzaldehyde



Herpetetrone

Figure 2 Chemical structures of representative anti-hepatitis B virus natural lignans.

LPRP-Et-97543 attenuated the nuclear expression of p65/p50 proteins from nuclear factor-kappaB (NF-κB) family and augmented the protein level of cytoplasmic IκBα without affecting their expression in HBV-nontransfected cells. Furthermore, LPRP-Et-97543 decreased the binding of NF-κB protein to CS1 site of HBV surface gene and suppressed CS1 element containing promoter activity in HBV-expressing cells. Nevertheless, HBV transfection markedly increases CS1 containing promoter activity in cells. Lastly, the inhibitory activity of LPRP-Et-97543 on HBV DNA replication was significantly reversed by transfection with the plasmid expressing p65 in HBV-positive cells. In conclusion, the feedback regulation of HBV gene expression and DNA replication may contribute to the anti-HBV effect of LPRP-Et-97543 by HBV proteins, which interferes with the NF-κB pathway[39].

Curcumin (a natural polyphenol) exhibited an inhibitory effect on HBV gene expression and replication in HBV-expressing cells by down-regulation of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α), a co-activator of HBV transcription, suggesting that curcumin may be a potential host-targeting agent for the treatment of HBV infection[40]. Additionally, since HBV is very sensitive to the metabolic regulator PGC-1α, targeting PGC-1a could be a potential strategy of anti-HBV therapy.

Neolancerin from *Swertia yunnanensis* showed an activity against the secretion of HBsAg with an IC_{50} value of 0.21 mmol/L and HBeAg with an IC_{50} value of 0.04 mmol/L; and norswertianolin, 1,8-dihydroxy-3,5-dimethoxyxanthone, neolancerin from *Swertia yunnanensis* showed a significant inhibitory effect on HBV DNA replication with IC_{50} values of 0.01, 0.07 and 0.09 mmol/L, respectively[13]. Six phenols (including m-hydroxybenzoic acid, phydroxybenzoic acid, m-hydroxy benzenmethanol, 3,4-dihydroxybenzoic acid, ethyl 3,4-dihydroxybenzoate and ethyl 2,5-dihydroxybenzoate) from *Swertia mussotii* exhibited activities inhibiting the secretion of HBsAg and HBeAg with IC_{50} values from 0.23 to 5.18 mmol/L, and HBV DNA replication with IC_{50} values from 0.06 to 2.62 mmol/L[41]. Ellagic acid was isolated from *Phyllanthus urinaria* and exhibited unique anti-HBV functions by blocking HBeAg secretion in HepG2 2.2.15 cells (IC_{50} = 0.07 mg/mL), suggesting

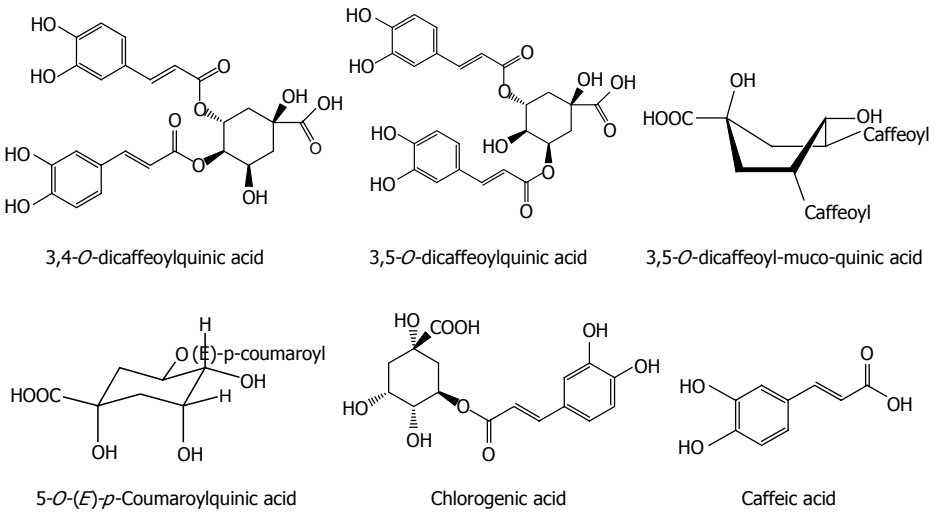


Figure 3 Chemical structures of representative anti-hepatitis B virus natural phenolic acids.

that it may be a potential agent for treatment of immune tolerance in individuals with HBV infection[42]. Protocatechuic aldehyde (PA) derived from *Salvia miltiorrhiza* appeared to down-regulate the HBsAg and HBeAg secretion as well as the HBV DNA release in HepG2.2.15 cells in a dose and time dependent manner; PA at doses of 25, 50, or 100 mg/kg (intraperitoneally, twice daily) also decreased viremia in ducks with DHBV infection[43]. Magnatriol B from *Streblus asper* exhibited moderate anti-HBV activities with IC_{50} values of 168.18 μmol/L (SI = 1.19) and 34.78 μmol/L (SI = 5.77) on HBsAg and HbeAg, respectively[29]. Mulberrofuran G from the root bark of *Morus alba L.* showed a moderate activity, inhibiting the replication of HBV DNA with an IC_{50} value of 3.99 μmol/L in HepG2.2.15 cells[44] (Figure 4).

LACTONES

Lactone is a naturaly component containing a cyclic ester of hydroxycarboxylic acid and also exhibits an antiviral activity. Costunolide and dehydrocostus lactone from the root of *Saussurea lappa* Clarks show a potent inhibitory effect on the HBsAg and HBeAg expression in Hep3B cells with IC_{50} values of 1.0 and 2.0 μmol/L, respectively. The suppression of HBsAg gene expression by the two compounds was mainly at the level of mRNA. Furthermore, the inhibitory activities of the two compounds on HBsAg and HBeAg were also observed in HepA2 cells[45]. 6-hydroxyl-7-methoxyl-coumarin from the heartwood of *Streblus asper* showed an anti-HBV effect, with an IC_{50} value of 29.60 μmol/L for HBsAg and an IC_{50} value of 46.41 μmol/L for HBeAg, respectively[23]. Erythrocentaurin (ET) as a C10-skeleton secoiridoid from *Swertia yunnanensis* inhibited the secretion of HBsAg with an IC_{50} value of 1.30 mmol/L and HBeAg with an IC_{50} value of 1.14 mmol/L, and HBV DNA replication with an IC_{50} value of 0.76 mmol/L; and a series of ET analogs with the alteration on the aldehyde group exhibited more potent anti-HBV agents[13,46].

Swerilactones E and F (two novel lactones with a phenyl group) from *Swertia mileensis* showed significant suppressive effects on the secretion of HBsAg with IC_{50} values of 0.22 and 0.70 mM and HBeAg with IC_{50} values of 0.52 and > 6.78 mmol/L in HepG 2.2.15 cells, respectively[47]. Artemisinin from *Artemisia annua* induced moderate inhibition on HBsAg secretion and HBV DNA replication with IC_{50} values of 55 and > 100 mmol/L, respectively[48]. Two pyranocoumarins clausenidin and nordentatin from *Clausena excavata* suppressed HBsAg in HepA2 cells[49]. Herpetosperin B from the seeds of *Herpetospermum caudigerum* showed an anti-HBV activity with HBsAg secretion by 33.1% at 200 μg/mL[50]. Swerilactones H-K from *Swertia mileensis* exhibit a potent anti-HBV activity against HBV DNA replication with IC_{50} values ranging from 1.53 to 5.34 μmol/L[51]. Swerilactones C and D from *Swertia mileensis* exhibited inhibitory activities in HepG 2.2.15 cells against the secretion of HBsAg (IC_{50} = 1.24 and 2.96 mmol/L, respectively) and HBeAg (IC_{50} = 0.77 and 1.47 mmol/L, respectively)[52] (Figure 5).

ALKALOIDS

Alkaloids are a class of naturally occurring compounds containing mostly basic nitrogen atoms and also include some related neutral and even weakly acidic compounds. Many alkaloids have potent pharmacological effects, such as quinine, pilocarpine, and atropine. Oxymatrine from the plant Kushen (*Sophora japonica*) has been used to treat patients with hepatitis B in China for decades with a confirmed safety and often is used to replace the nucleoside analogues in order to decrease the emergence of treatment-resistant HBV mutants[53]. Monotherapy with oxymatrine (orally, for twelve months) decreased blood HBeAg by 70% and HBV DNA by 96% in

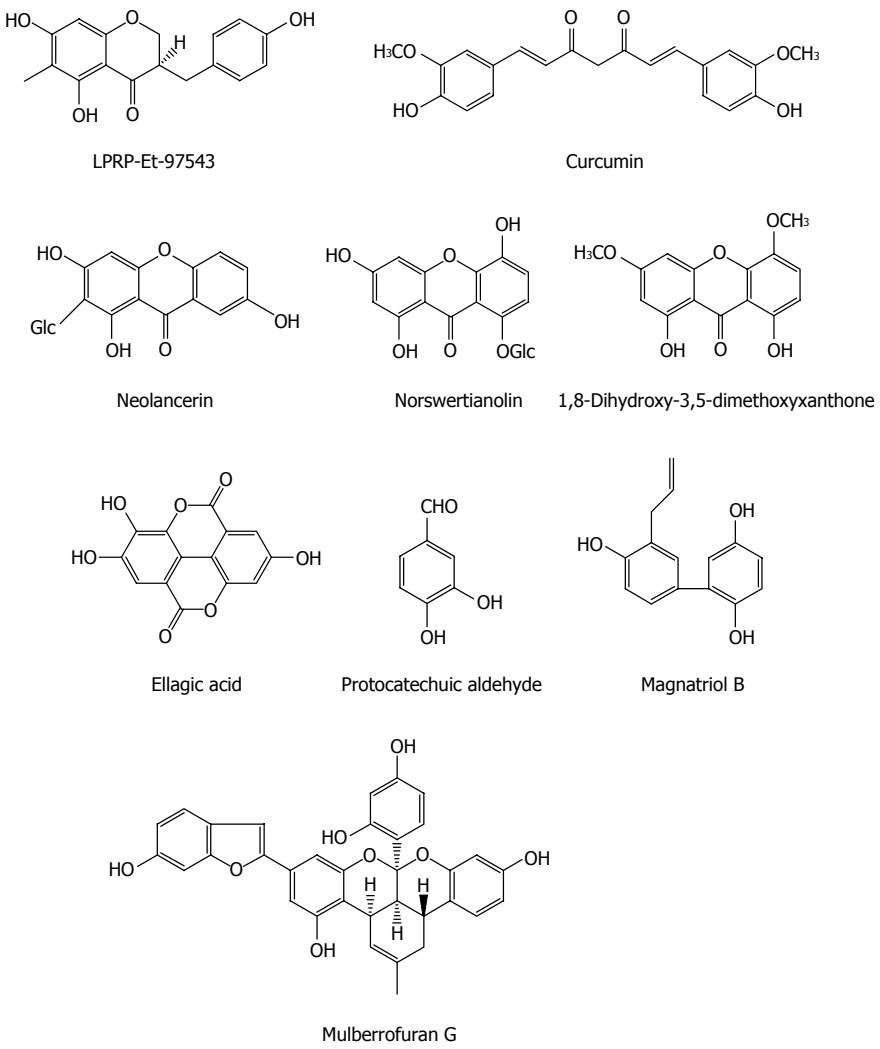


Figure 4 Chemical structures of representative anti-hepatitis B virus natural polyphenols.

patients with chronic hepatitis B (CHB) resistant to lamivudine (n = 17), equal to its efficacy in treatment-naïve CHB cohort (n = 20)[54]. Oxymatrine significantly down-regulates the mRNA expression of host heat-stress cognate 70 (Hsc70) at the post transcriptional level through destabilizing Hsc70 mRNA, and then inhibits the replication of HBV and exhibits anti-HBV efficacy[55].

Cepharanthine is a natural biscoclaurine alkaloid extracted from *Stephania cepharantha* Hayata. Cepharanthine hydrochloride (CH), as a natural alkaloid-derived compound, suppressed HBeAg production and HBV DNA replication by either wild-type or lamivudine-resistant HBV clinical isolates in a dose dependent manner and down-regulated significantly the Hsc70 mRNA levels, suggesting that its activity could be associated with its inhibitory effect on host Hsc70[56]. Squalamine from the sea lamprey (*Petromyzon marinus*) and the dogfish shark (*Squalus acanthias*) possesses a broad-spectrum antiviral effect on human viruses including HBV. Enveloped DNA and RNA viruses are proved to be susceptible, suggesting that it has a broad-spectrum antiviral activity. The antiviral mechanism of squalamine is likely based on its capacity to neutralize the negative electrostatic surface charge of intracellular membranes that renders the host cell less effective in the process of viral replication[57]. Dauricumidine from *Hypserpa nitida* Miers showed an IC_{50} value of 0.450 mM (SI = 4.13) on HBsAg secretion in HepG2.2.15 cells[58]. N,N-dimethyltryptamine N12-oxide from liver-protective medicinal plant *Evodia fargesii Dode* (Rutaceae) had a potent suppressive effect on HBV DNA replication (IC_{50} = 17.6 μmol/L, SI > 5.7) in HepG2.2.15 cells[59]. Dihydrochelerythrine isolated from *Corydalis saxicola* Bunting exhibited the strongest effect on HBsAg and HBeAg secretion with an IC_{50} value < 0.05 mM and SI > 3.5, respectively[60]. Dehydrocavidine, dehydroapocavidine and dehydroisoapocavidine from *Corydalis saxicola* Bunting (Papaveraceae) exhibited a suppressive activity against the secretion of HBsAg and HBeAg in Hep2.2.15 cells[61] (Figure 6).

FLAVONOIDS

Flavonoids are a group of polyphenolic compounds

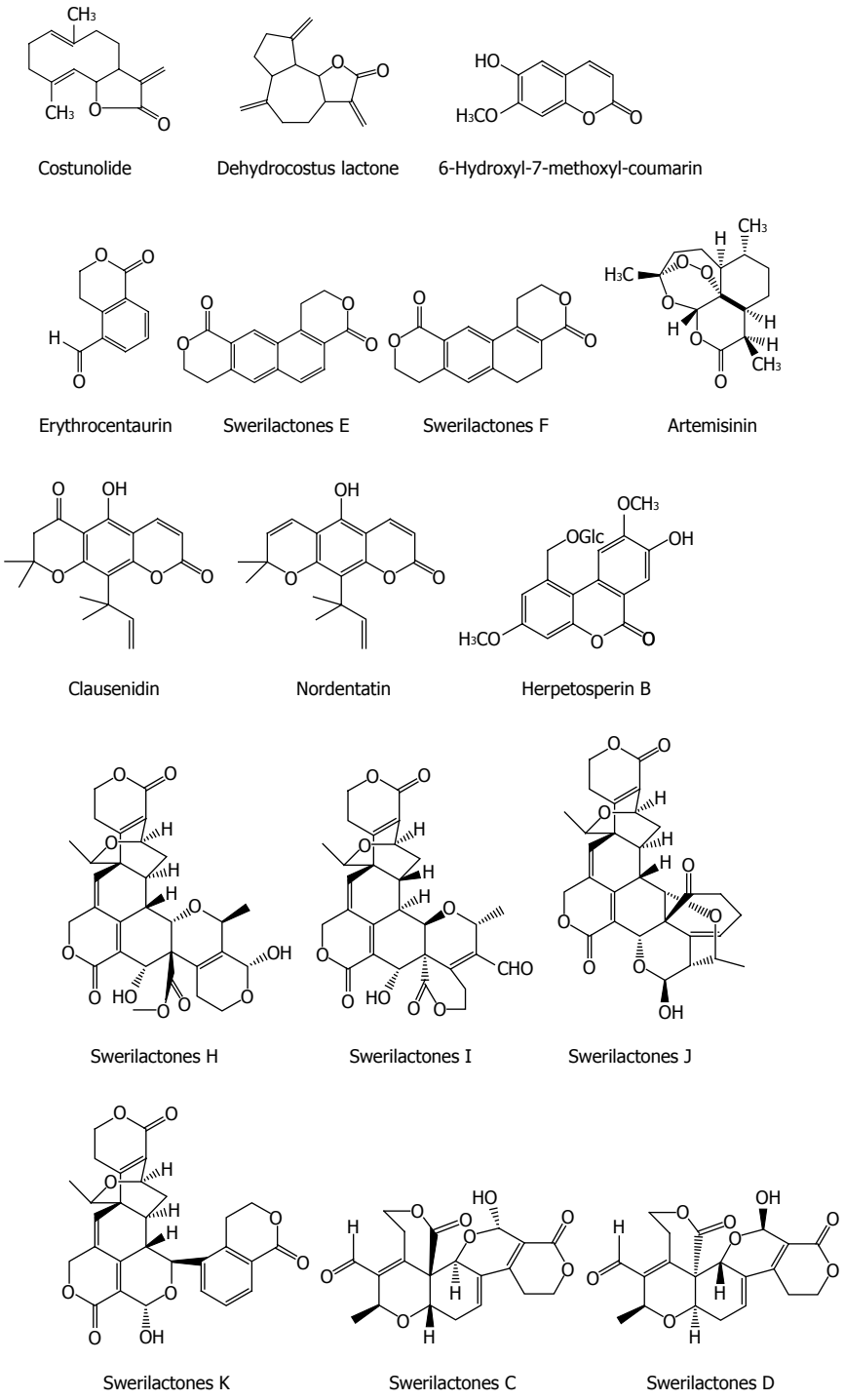


Figure 5 Chemical structures of representative anti-hepatitis B virus natural lactones.

containing a 15-carbon skeleton (C6-C3-C6), which consists of a heterocyclic ring (C) and two phenyl rings (A and B). Flavonoids have a wide range of pharmacological effects including antiviral activity. Epigallocatechin-3-gallate (EGCG) from green tea suppressed HBV entry into immortalized human primary hepatocytes at a concentration of 50 μmol/L by more than 80%[62]. EGCG effectively inhibited the HBsAg and HBeAg secretion in HepG2 2.2.15 cells in a dose and time dependent manner, whereas its effect was stronger than that of lamivudine; EGCG also reduced the extracellular HBV DNA level[63]. Sodium taurocholate cotransporting polypeptide (NTCP), a new-found receptor for HBV, is expressed in DMSO-differentiated HuS-E/2 cells. During HBV inoculation, EGCG significantly suppressed viral infection in both HA-NTCP-expressing Huh7 cells and DMSO-differentiated HuS-E/2 cells. Meanwhile, EGCG induced clathrin-dependent endocytosis of NTCP from the plasma membrane followed by lysosomal degradation. Additionally, EGCG suppressed the clathrin-mediated endocytosis of transferrin. However, no inhibitory

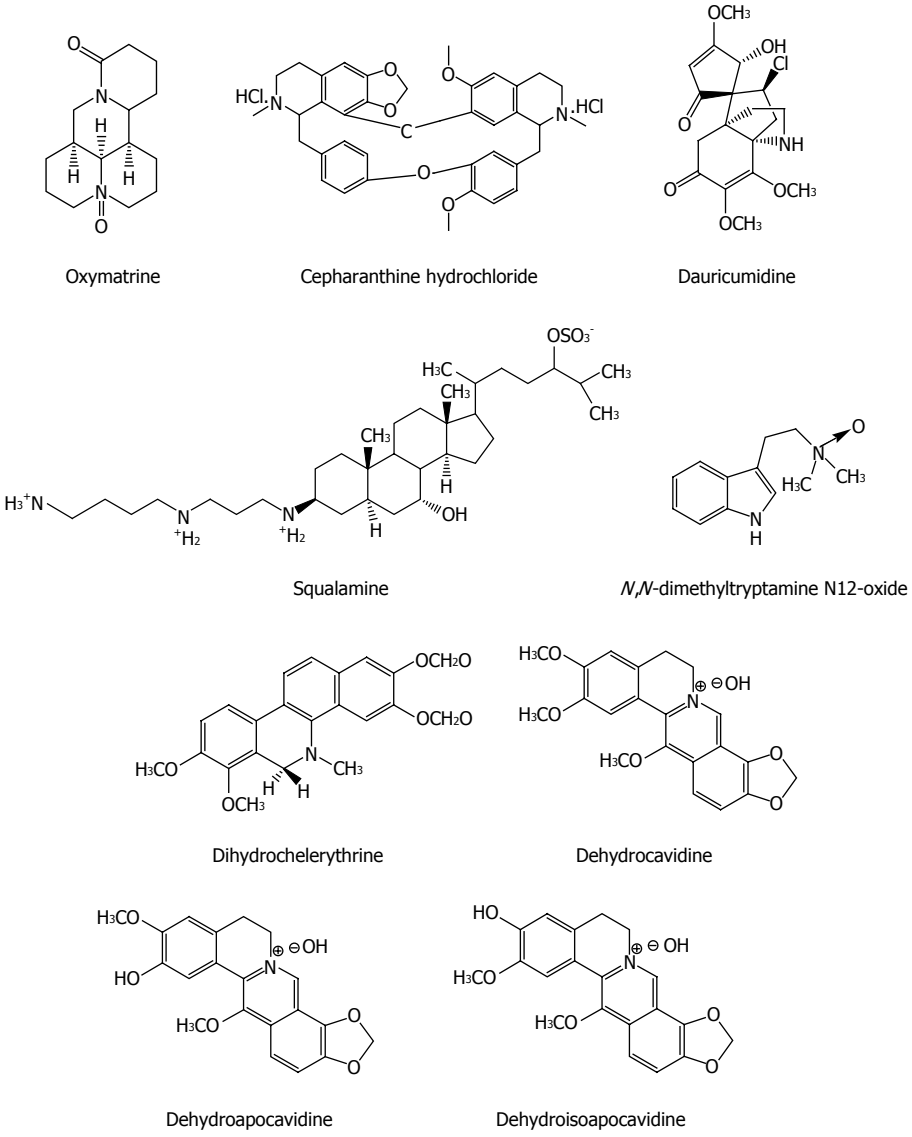


Figure 6 Chemical structures of representative anti-hepatitis B virus natural alkaloids.

effect of EGCG was observed on HBV virion secretion or genome replication in cells *in vitro*. Furthermore, EGCG also unaltered the HBV entry factor expression and virion characteristic. Finally, the potent inhibitory effect of EGCG on HBV entry was validated using four different genotypes, A to D, suggesting that it could be a potential agent for prevention of HBV reinfection[62].

Luteolin and isovitexin from *Swertia yunnanensis* showed inhibitory effects on the secretion of HBsAg (IC_{50} values of 0.10 and < 0.03 mmol/L) and HBeAg (IC_{50} values of 1.51 and 0.23 mmol/L), and exhibited a marked suppressive effect on HBV DNA replication (IC_{50} values < 0.01 and 0.05 mmol/L)[13]. Isoorientin from *Swertia mussotii* displayed significant anti-HBV activities against HBsAg and HBeAg secretion with IC_{50} values of 0.79 and 1.12 mmol/L, as well as HBV DNA replication with an IC_{50} value of 0.02 mmol/L[41]. Wogonin from *Scutellaria radix* effectively inhibited the HBV antigen secretion with an IC_{50} value of 4 mg/mL for both HBsAg and HBeAg and also reduced HBV DNA level in hepG2.2.15 cells. DHBV DNA polymerase was dramatically suppressed by wogonin with an IC_{50} value of 0.57 mg/mL. In ducks with DHBV infection, wogonin decreased the plasma level of DHBV DNA with a 50% effective dose (ED_{50}) of 5 mg/kg. In human HBV-transgenic mice, wogonin significantly reduced plasma HBsAg level[64]. Robustaflavone isolated from *Rhus succedanea* exhibited a potent inhibitory effect on HBV replication in HepG2.2.15 cells, with a 50% effective concentration (EC_{50}) of 0.25 mM, and an SI of 153. Robustaflavone hexaacetate suppressed HBV replication with an EC_{50} of 0.73 mmol/L[65] (Figure 7).

OTHERS

Except for the above-mentioned chemical classes, some other natural components also have anti-HBV activities. *p*-Hydroxyacetophenone (*p*-HAP) from *Artemisia morrisonensis* exhibited an inhibitory activity on HBsAg secretion and HBV DNA replication with

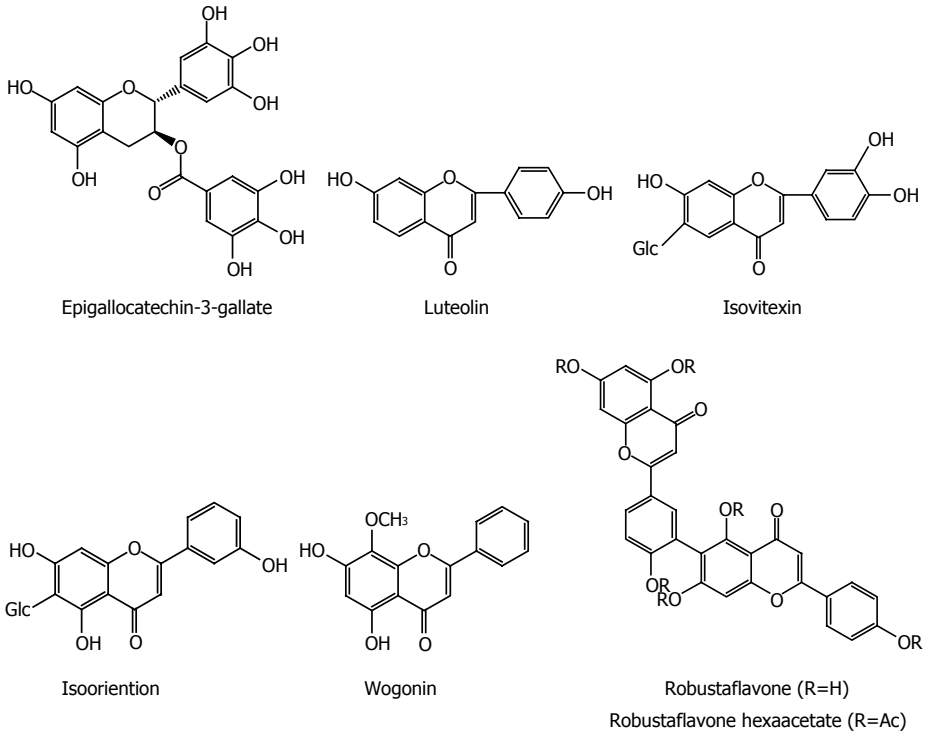


Figure 7 Chemical structures of representative anti-hepatitis B virus natural flavonoids.

IC_{50} values of 785.7 and 306.4 μmol/L, respectively. Additionally, 6'-*O*-Caffeoyl-*p*-hydroxy-acetophenone-4-*O*-β-*D*-glucopyranoside from A. capillaris possessed a significant suppressive effect on HBV DNA replication with an IC_{50} value of 8.0 μmol/L[66]. In the HBV-transfected Huh7 cells, the 2.4 kb preS RNA of HBV surface gene enhanced markedly relative to the 2.1 kb S RNA with p-HAP. p-HAP markedly augmented the promoter activity of HBV preS and specifically reduced endoplasmic reticulum (ER) stress related glucose-regulated protein 78 RNA/protein levels, but not those of glucose-regulated protein 94, in treated Huh7 cells. *p*-HAP also led to a marked intracellular accumulation of virus. Furthermore, treatment with thapsigargin (ER chaperone inducer) relieved the suppressive effect of *p*-HAP on the HBV DNA levels in the supernatant of HBV-expressed cells. Hence, the anti-HBV mechanism of *p*-HAP could involve the regulation of HBV surface gene expression and block the secretion of virion by interference with the ER stress pathway[67].

β-Thujaplicinol from the heartwood of Western Red Cedar trees (*Thuja plicata*, *Thuja occidentalis* and *Chamaecyparis obtusaI*) inhibited RNAseHs from HBV genotypes D and H in biochemical assays with IC_{50} values of 5.9 and 2.3 μmol/L, respectively. It blocked replication of HBV genotypes A and D in culture by inhibiting the RNAseH activity with an estimated EC_{50} of 5 μmol/L and a 50% cytotoxic concentration (CC_{50}) of 10.1 mmol/L[68]. Chrysophanol 8-*O*-*β*-*D*-glucoside from *Rheum palmatum L.* showed a significant inhibitory effect on HBV DNA production and antigen expression with an IC_{50} value of 36.98 mg/mL on HBV DNA inhibition, and effectively suppressed HBV DNA polymerase activity[69]. 7-Dehydroxyl-zinniol from the culture of *Alternaria solani*, an endophytic fungal strain residing in the roots of *Aconitum transsectum* showed a moderate anti-HBV activity[70] (Figure 8).

CONCLUSION

Despite that several anti-HBV drugs and genetic engineering vaccines are available for patients with HBV, HBV infection is still a severe public health problem in the world. In fact, no effective drugs or therapeutic methods can cure hepatitis B so far. Therefore, it is urgent affairs to further intensify research and development of anti-HBV new drugs, especially non-nucleoside agents. Natural products with enormous molecular complexity and diversity offer a large opportunity for finding novel antiviral lead compounds or good candidates with unique anti-HBV mechanisms. In this review, the natural products with anti-HBV activity are classified and analysed according to their structure types including terpenes, lignans, phenolic acids, polyphenols, lactones, alkaloids and flavonoids. These anti-HBV natural products can be cited as promising leads or candidates.

The anti-HBV mechanisms of action of the compounds described above involve multiple aspects, and several natural compounds exhibit special antiviral characteristics and novel mode of action. Betulinic acid (a triterpene) from *Pulsatilla chinensis* reduces HBV replication by inhibiting the expression of SOD2 with subsequent overgeneration of mitochondrial ROS, with promising HBV clearance both in HBV-infected hepatocytes and in HBV-transgenic mice[7].

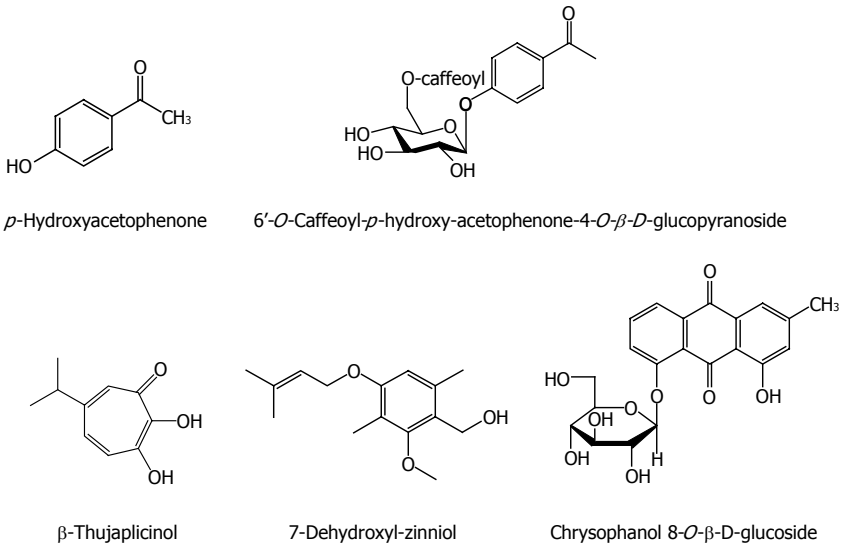


Figure 8 Chemical structures of other anti-hepatitis B virus natural products.

Helioxanthin (a lignan) from the heartwood of *Taiwania cryptomerioides Hayata* is a promising anti-HBV agent and acts through liver-specific transcriptional machinery for viral CP to suppress the gene expression of HBV and the production of viral particles *in vitro*[25]. The anti-HBV effects of 3,4-*O*-dicaffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid (a phenolic acid) from *Laggera alata* are probably associated with the up-regulation of HO-1 by decreasing HBV core protein stability, which blocks nuclear HBV cccDNA refill in HepG2.2.15 cells[35,36]. The mechanism of HBV inhibition by LPRP-Et-97543 (a polyphenol) from *Liriope platyphylla* might involve feedback control of the gene expression and DNA replication of HBV by viral proteins, which interferes with the NF-κB pathway in HBV-transfected Huh7 cells[39]. Curcumin (a polyphenol) suppresses the gene expression and DNA replication of HBV by down-regulation of PGC-1α, a starvation-induced protein that initiates the gluconeogenesis cascade and that can coactivate robustly HBV transcription in HBV expressing cells[40]. β-Thujaplicinol (a polyphenol) from the heartwood of Western Red Cedar trees inhibits HBV replication by blocking the activity of viral ribonuclease H *in vitro*[68]. Oxymatrine (a alkaloid) from *Sophora japonica* and Cepharanthine (a alkaloid) from *Stephania cepharantha* down-regulate Hsc70 mRNA expression at the post transcriptional level through destabilizing Hsc70 mRNA, and then inhibits HBV replication in cells[55,56]. Epigallocatechin-3-gallate (a flavonoid) from green tea, as a new HBV entry inhibitor, induced clathrin-mediated endocytosis of NTCP-HBV from the plasma membrane followed by lysosomal degradation and suppressed the clathrin-mediated endocytosis of transferrin *in vitro*[62]. The *in vitro* mechanism of HBV inhibition by p-Hydroxyacetophenone from *Artemisia morrisonensis* may involve the regulation of HBV surface gene expression and blocking the secretion of virions by interference with the ER stress signaling pathway[67].

In general, there are not many reports of novel mode of action or new targets of anti-HBV natural products. Additionally, although many natural products are reported to exhibit potent anti-HBV effects to date, they are limited to cell-based or transgenic mice-based studies. Unfortunatley, none of these have been added into the existing list of anti-HBV drugs. Given that many of these naturally originated compounds with various skeletons and diverse biological activities discussed above are already available, future efforts should be devoted to optimize and develop these lead compounds into potent anti-HBV agents for clinical application. Continued screening of natural products with anti-HBV activities may find some novel skeletons that possess new mechanism of action, but it may be difficult to directly lead to the discovery of any clinical candidate compounds.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Advances in computed tomography and magnetic resonance imaging of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. Imaging is important for establishing a diagnosis of HCC and early diagnosis is imperative as several potentially curative treatments are available when HCC is small. Hepatocarcinogenesis occurs in a stepwise manner on a background of chronic liver disease or cirrhosis wherein multiple genes are altered resulting in a range of cirrhosis-associated nodules. This progression is related to increased cellularity, neovascularity and size of the nodule. An understanding of the stepwise progression may aid in early diagnosis. Dynamic and multiphase contrast-enhanced computed tomography and magnetic resonance imaging still form the cornerstone in the diagnosis of HCC. An overview of the current diagnostic standards of HCC in accordance to the more common practicing guidelines and their differences will be reviewed. Ancillary features contribute to diagnostic confidence and has been incorporated into the more recent Liver Imaging Reporting and Data System. The use of hepatocyte-specific contrast agents is increasing and gradually changing the standard of diagnosis of HCC; the most significant benefit being the lack of uptake in the hepatocyte phase in the earlier stages of HCC progression. An outline of supplementary techniques in the imaging of HCC will also be reviewed.

Key words: Hepatocellular carcinoma; Computed tomography; Magnetic resonance imaging; Contrast agent; Cirrhosis; Ancillary features

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Core tip: Imaging is important for establishing a diagnosis of hepatocellular carcinoma (HCC) and an understanding of the stepwise progression of hepatocarcinogenesis may aid in early diagnosis. Dynamic and multiphase contrast-enhanced computed tomography and magnetic resonance imaging still form the cornerstone in the diagnosis of HCC. An overview of the current diagnostic standards of HCC in accordance to the more common practicing guidelines and their differences will be reviewed. Various ancillary

features, use of hepatocyte-specific contrast agents and supplementary imaging techniques also help to increase diagnostic confidence and will be reviewed.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. It ranks sixth in cancer incidence and third in cancer mortality worldwide[1]. It is the most prevalent liver cancer with up to three-quarter of cases in the world occurring in Asia due to the high prevalence of chronic viral hepatitis B[2]. Patients diagnosed with HCC generally have a poor prognosis due to the aggressive nature of the disease[3]. Early diagnosis of HCC is imperative as several potentially curative treatments are available, especially when the lesion is small.

Regular surveillance of patients is instituted for early detection of HCC in patients with chronic liver disease and particularly in those with advanced liver fibrosis. Screening involves clinical examination, serum analysis of liver function and tumour antigens such as alpha-fetoprotein (AFP) and imaging. Although AFP is not specific for HCC and may give false positive results in the setting of hepatitis and fibrosis, it is still useful in monitoring of the disease process in combination with imaging[4]. Non-invasive diagnosis with imaging is currently the preferred method and several guidelines are available to aid in diagnosis and they all endorse arterial enhancement followed by washout in the diagnosis of HCC (Figure 1). Dynamic and multiphase contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) form the cornerstone of diagnosis in HCC. This review presents an overview of the current diagnostic standards of HCC in accordance to the more common practicing guidelines as well as the use of ancillary features and hepatocyte-specific contrast agents in the diagnosis of HCC. An outline of supplementary techniques in the imaging of HCC will also be reviewed.

EPIDEMIOLOGY

HCC is associated with chronic liver disease and cirrhosis irrespective of its etiology. It has been shown that only about 10% of HCCs develop in non-cirrhotic livers[5]. The incidence of HCC has been increasing, with chronic hepatitis B and C infections being major contributory factors worldwide[6]. Apart from chronic viral infection, several lifestyle factors contribute to the development of HCC. These include excessive alcohol consumption, obesity, diabetes and intake of aflatoxin-contaminated foods[7]. Greater than 90% of HCC cases develop in chronically inflamed liver as a result of viral hepatitis and alcohol abuse[8]. Obesity and diabetes are associated with development of non-alcoholic fatty liver disease (NAFLD)[9]. Insulin resistance and the resulting inflammatory cascade together with the development of non-alcoholic steatohepatitis (NASH) appear to encourage hepatocarcinogenesis[10]. Cigarette smoking is regarded as a co-factor in the development of HCC[11]. Hepatocarcinogenesis also increases in the setting of HIV infection[12]. Lastly, genetic conditions such as haemochromatosis, glycogen storage disease type 1, alpha 1-antitrypsin deficiency are all associated with increased risk of HCC, most frequently on a background of cirrhosis[13].

PATHOGENESIS

In patients with chronic liver disease, HCC typically develops in a stepwise manner wherein multiple genes are altered. Chronic inflammation and regeneration of hepatocytes are underlying causes; it results in damage to the DNA of regenerating hepatocytes hence increasing the chance of gene alterations associated with carcinogenesis[14]. The currently accepted nomenclature for stepwise carcinogenesis of HCC is: regenerative nodule (RN); low-grade dysplastic nodule (DNI); high-grade dysplastic nodule (DNII); early and progressed HCC[15-17].

Regenerative nodules

These are typically well-defined rounded regions of the cirrhotic parenchyma surrounded by scar tissue[18]. RNs are essentially phenotypically normal and are usually considered benign lesions[19]. Relative to background parenchyma, they are typically isoattenuating on unenhanced CT, T1, T2 and diffusion weighted (DWI) MR imaging[20,21] (Figure 2). On occasion, they may be T1 hyperintense and T2 hypointense, similar to dysplastic nodules[22]. With intravenous extracellular contrast injection, most RN enhance to the same degree as adjacent liver parenchyma or show slightly less enhancement, hence, they may appear as mildly hypoattenuating nodules relative to enhancing fibrosis in the portal venous phase[23] (Figure 3).

Dysplastic nodules

These are nodular lesions that differ macroscopically and microscopically from background parenchyma[24]. They are classified as low or high grade depending on the presence of cytologic and architectural aberrations[25]. DNI resemble RN histologically except that they contain unpaired arteries and clone-like populations[24,25]. On the other hand, DNII show features similar to that of a well-differentiated HCC. They demonstrate cellular atypia with clone-like features, expansile subnodules and

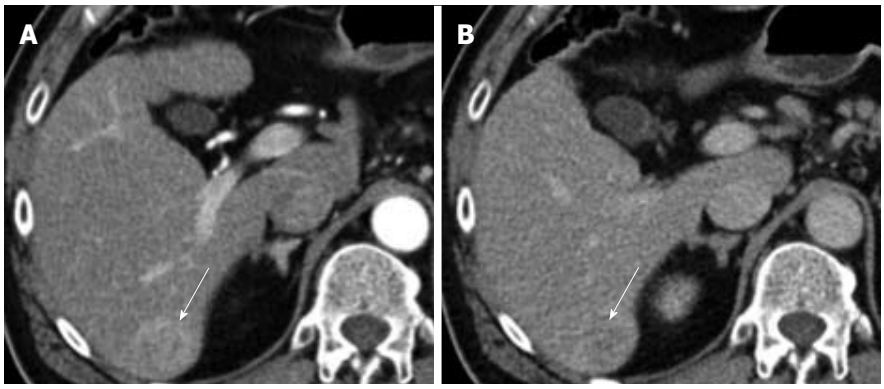

Figure 1 Typical features of arterial enhancement (A) with washout in the portal venous phase (B) is noted in segment 6 in keeping with histological-proven hepatocellular carcinoma.

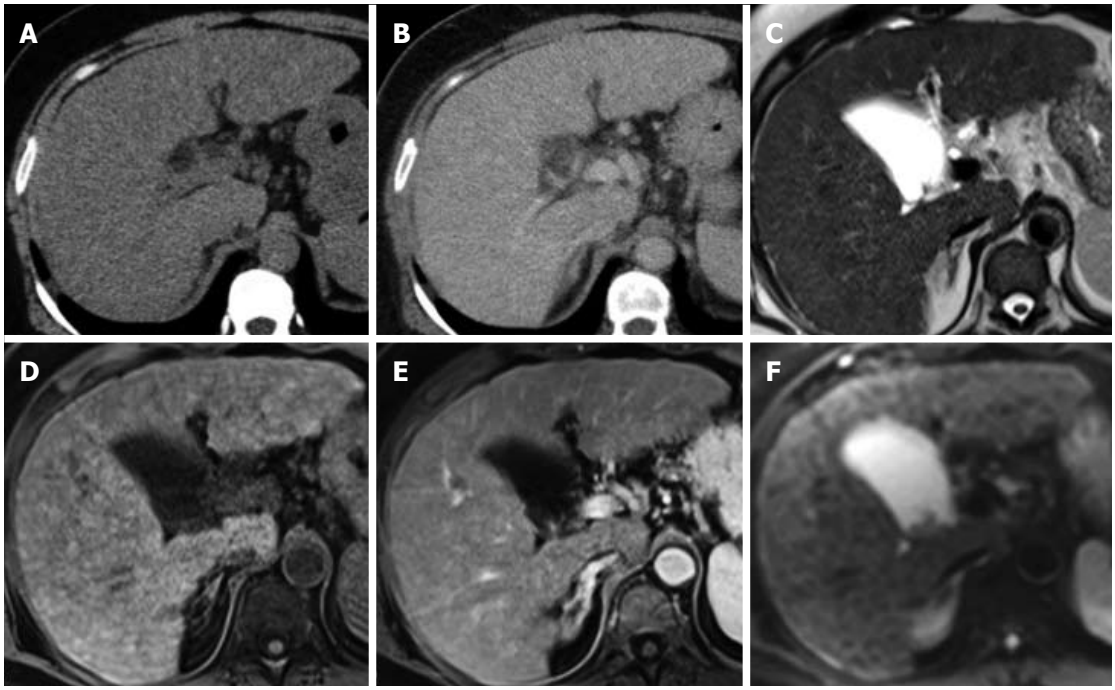

Figure 2 The liver demonstrates a nodular outline consistent with cirrhosis and multiple small regenerative nodules that are isodense on unenhanced (A) and portal venous phase (B) on computed tomography, predominantly isointense on T2W (C) and T1W (D) sequences with no evidence of arterial enhancement (E) or restricted diffusion (F).

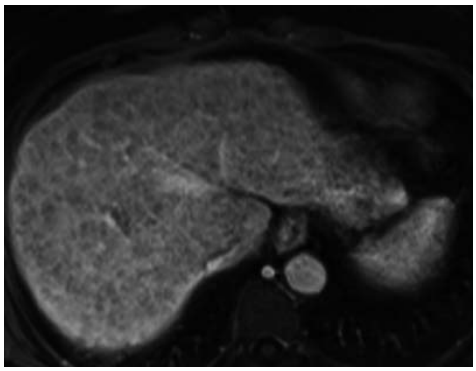

Figure 3 Multiple regenerative nodules in the portal venous phase may appear mildly hypoattenuating relative to enhancing fibrosis.

architectural alterations[25,26]. Some DNII may contain subnodules of HCC resulting in the nodule-in-nodule appearance[27]. On CT, most DN are hypo- or isodense in the arterial, portal venous and delayed phases[28]. They are typically T1 hyperintense and iso- to hypointense on T2 imaging[23] (Figure 4). Some, especially DNII may contain intracellular fat resulting in signal loss on out-of-phase images[29]. Unlike HCC, DN are almost never T2 hyperintense or show restricted diffusion[30,31] (Figure 4).

Early HCC

Early HCC is likened to carcinoma-*in-situ* of other organs[32]. They rarely exceed 2 cm and unlike progressed HCC which displaces and destroys sur-

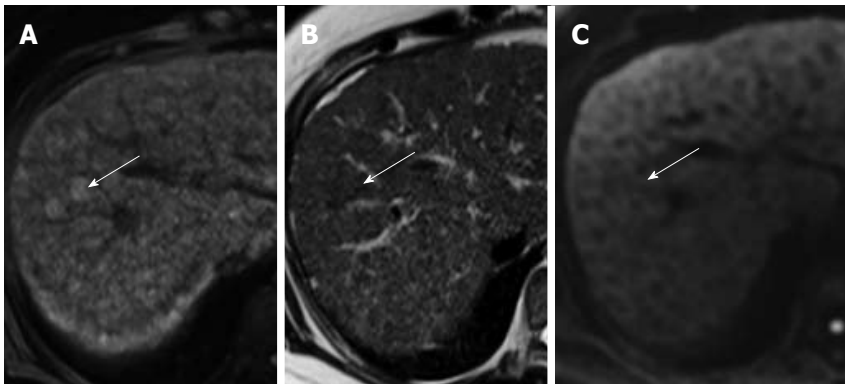

Figure 4 Dysplastic nodules may appears hyperintense on T1W (A), iso-hypointense on T2W (B) but do not show restricted diffusion (C).

rounding liver parenchyma, early HCCs expand by gradually replacing the parenchyma[17]. The main distinguishing characteristic between a DNII and early HCC is the presence of stromal invasion in the latter which is defined as infiltration of tumour cells into fibrous tissue surrounding portal tracts[25].

Progressed HCC

Theses nodules are overtly malignant with propensity to invade vessels and metastasize. Lesions smaller than 2 cm are typically distinctly nodular with well-defined margins; they grow by expanding into and compressing surrounding parenchyma resulting in formation of a pseudocapsule[17]. Lesions larger than 2 cm demonstrate a more aggressive behaviour. A mosaic pattern is characteristic which is defined by the presence of several internal subnodules separated by fibrous septa as well as areas of necrosis, haemorrhage and occasionally fatty metamorphosis[33].

CURRENT DIAGNOSTIC STANDARDS OF HCC ACCORDING TO EXISTING GUIDELINES

In oncology, the diagnosis of malignancy usually necessitates tissue sampling prior to determination of treatment approach. Characterisation of HCC however, is an exception as a non-invasive diagnosis can be attained with imaging in high-risk patient populations[2,34,35]. The more widely used guidelines are the European Association for the Study of the Liver (EASL)[34], American Association for the Study of Liver Disease (AASLD)[35] and the Asian Pacific Society for the Study of the Liver (APASL)[2]. The hallmark diagnostic characteristics of HCC are arterial enhancement followed by portal venous and/or delayed phase washout[36-38] (Figures 1 and 5), this is common to all three guidelines. Comparative studies for CT and MR imaging using extracellular contrast agents found higher sensitivities with MR imaging[39,40]. The sensitivity of MRI for nodular HCC of all sizes is 77%-100% while that of CT is 68%-91%[34,35,41,42]. The size of the lesion is an important determinant in diagnosis; for lesions larger than 2 cm, the sensitivity is close to 100% for both modalities but drops to 45%-80% with MRI and 40%-75% with CT for lesions measuring 1-2 cm[40,43].

Both EASL and AASLD stratify lesions according to size; < 1 cm, 1-2 cm and > 2 cm for EASL and < 1 cm and > 1 cm for AASLD. Both guidelines deem less than 1 cm lesions as too small for characterisation and recommend follow-up. The diagnosis of HCC in lesions larger than 2 cm requires only a single imaging modality when the hallmark enhancement characteristics are present. Another imaging technique should be performed when enhancement characteristics are atypical. These guidelines differ with respect to lesions between 1-2 cm; the AASLD recommends the same approaches as for lesions larger than 2cm whereas EASL recommends the presence of typical enhancement characteristics on two imaging modalities. Both EASL and AASLD recommend biopsy in patients with lesions that do not fit in the above imaging criteria. Unlike EASL and AASLD, APASL does not stratify lesions according to size. Also, the APASL acknowledges the use of contrast-enhanced ultrasound (CEUS) to depict hypervascularity in lesions hypovascular on CT or MRI. When a defect is observed in the Kupffer phase on CEUS, it is diagnosed as HCC. The Kupffer phase also known as the post-vascular phase which occurs 20 min after injection and implies the presence of Kupffer cells which are present in non-neoplastic liver parenchyma and reduced in HCC[44]. If this defect is not observed, close follow-up is recommended.

The Liver Imaging Reporting and Data System (Li-RADS)[45] was introduced relatively recently by the American College of Radiology. The aim of this system was to standardize terminology and criteria in reporting of liver lesions in chronic liver disease. Each lesion is assigned a category ranging from L1 to L5, with each category denoting a higher probability of HCC. Unlike the above mentioned guidelines, Li-

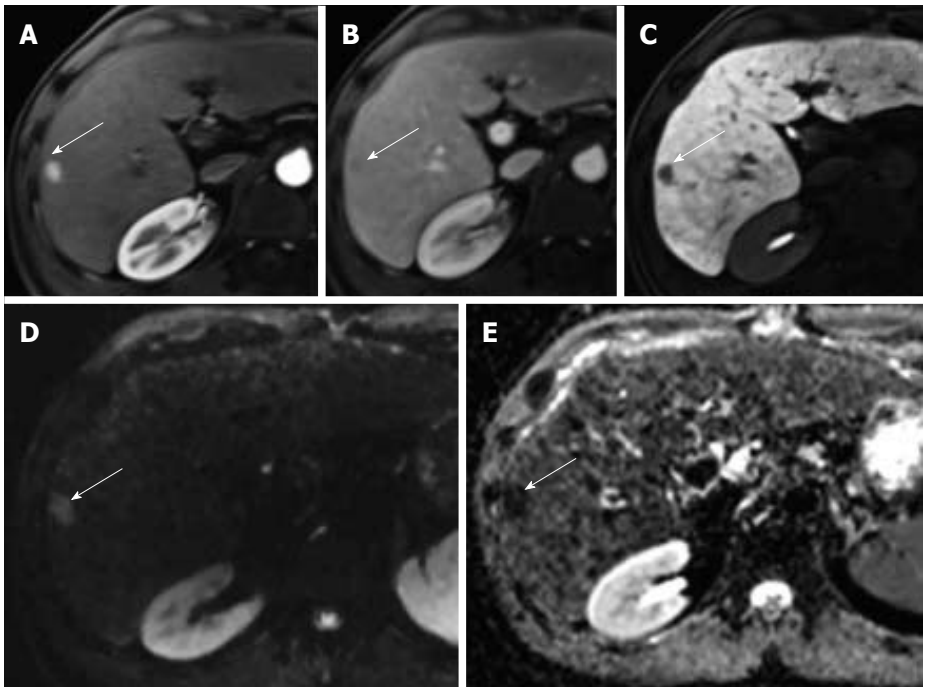

Figure 5 Typical characteristics of a hepatocellular carcinoma. A small lesion in segment 6 demonstrates arterial enhancement (A), washout in the portal venous phase (B), hypointensity in the hepatobiliary phase (C) and restricted diffusion [hyperintense on DWI (D) and hypointense on ADC (E)].

Table 1 The Liver Imaging Reporting and Data System

		Arterial phase hypo- or iso-enhancement		Arterial phase hyper-enhancement		
Diameter (mm)		< 20	⩾ 20	< 10	10-19	⩾ 20
Washout	None	L3	L3	L3	L3	L4
Capsule formation	One	L3	L4	L4	L4/L5	L5
Threshold growth	⩾ Two	L4	L4	L4	L5	L5

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RADS takes into account ancillary features. The diagnosis of HCC is established by a combination of major signs including: arterial phase enhancement, lesion size, washout, capsule formation and threshold growth (Table 1). Ancillary features are then applied to upgrade or downgrade the initial classification.

ANCILLARY FEATURES

A substantial proportion of HCCs do not demonstrate the typical arterial enhancement with subsequent washout pattern. It has been shown that up to 40% of HCC lack arterial phase enhancement[34], these are largely early or poorly-differentiated infiltrative HCCs[46,47]. Also, 40%-60% of small HCCs do not demonstrate subsequent washout[48,49]. Hence, several ancillary signs have been described, most of which are better depicted with MRI. It is important to emphasise that these features individually are not specific for HCC, but their presence increases diagnostic probability.

Restricted diffusion

DWI assesses molecular water motion within tissues and this information is acquired by applying balanced gradients to T2-weighted sequences. The degree of diffusion weighting can be altered by changing the *b* value, an acquisition parameter. With DWI, signal intensity from stationary water molecules is preserved whilst those that are in motion lose signal intensity depending on the degree of motion from their original position at the time of signal acquisition. Diffusion restriction is more prominent in malignant than in benign tumours[50]; the combination of high cellularity and intact cell membranes restrict the motion of water molecules resulting in hyperintensity on diffusion weighted imaging (DWI) and reduction in apparent diffusion coefficient (ADC) maps. DWI is particularly useful in the initial screening of the liver as nearly 70%-95% of HCCs can appear hyperintense[51-53], particularly using low *b* values[54]. The presence of restricted diffusion is found to be especially useful in the characterisation of small lesions[55,56] (Figure 5). Intermediate or poorly-differentiated HCCs are more often hyperintense on DWI than well-differentiated HCC[16]. In addition, restricted diffusion may be useful in the diagnosis of bland versus tumour thrombus[16].

Intralesional fat

The presence of fat in a focal liver lesion is better appreciated on MRI than on CT. The presence of fat is depicted as signal drop-out in the opposed-phase images (Figure 6). In chronic liver disease, a fat-contained tumour is highly suggestive of HCC[42],

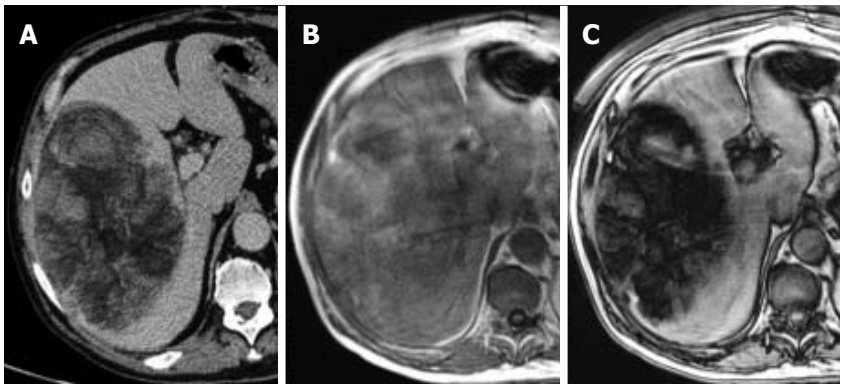

Figure 6 A large hepatocellular carcinoma in the right lobe of the liver demonstrates fat attenuation on non-contrast enhanced computed tomography (A), and loss of signal in the in- (B) and opposed-phase (C) images indicative of fat.

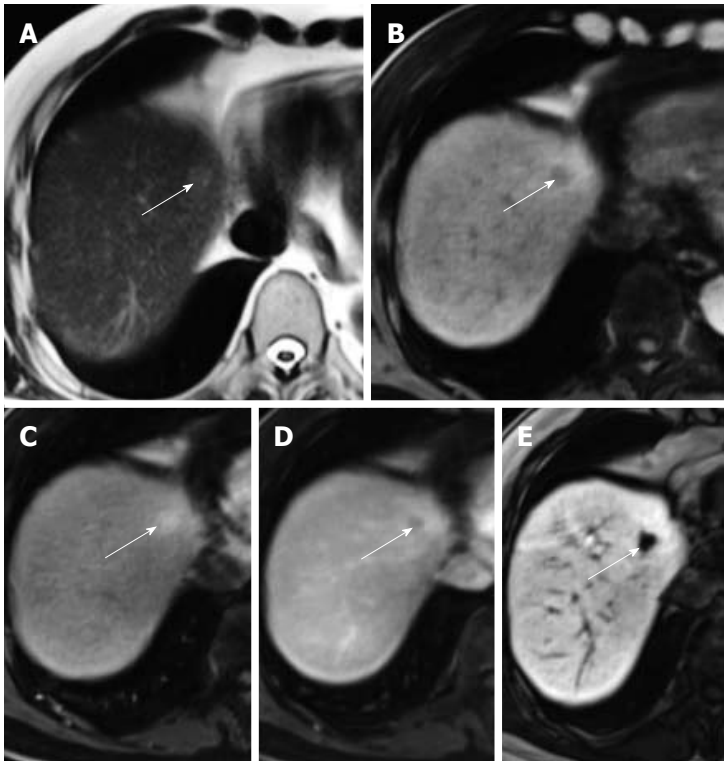

Figure 7 A small hepatocellular carcinoma demonstrates mild T2W hyperintensity (A), T1W hypointensity (B), arterial enhancement (C), portal venous phase washout (D) and hypointensity on the 20 min hepatobiliary phase (E) after injection with Gd-EOB-DTPA.

however, benign fat-containing regenerative nodules may also be seen[16]. Intralesional fat is more commonly seen in early as opposed to progressed HCC, with better prognosis associated with fat-contained HCC[55].

Mild to moderate T2 signal intensity

On MRI, the presence of mild to moderate T2 signal intensity is more often seen in HCCs (Figure 7). Markedly T2 hyperintense lesions are more likely to represent benign lesions such as cysts and haemangiomas, whereas T2 hypointense lesions may represent iron deposition in the nodules. Like the presence of intralesional fat, the degree of T2 signal intensity may have prognostic implications; many well-differentiated HCCs are found to be hypo- or isointense[56].

Mosaic pattern

The variable tissue components of HCC account for this mosaic pattern; enhancing areas indicate viable tumour cells and low attenuation foci represent necrosis, fibrosis or hemorrhage[33]. Most large HCCs present with this pattern and it is regarded as fairly specific (Figure 8). Since it is found primarily in large lesions, the utility of this ancillary sign is probably of less utility in the characterisation of small HCCs.

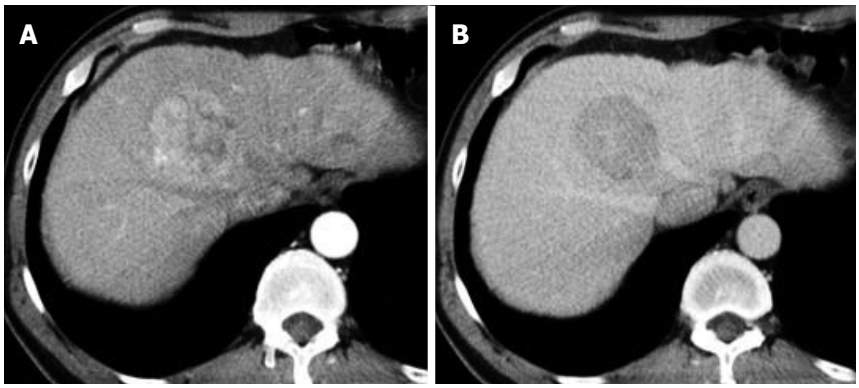

Figure 8 Mosaic attenuation is demonstrated on the arterial phase sequence (A) in this relatively large hepatocellular carcinoma followed by washout (B).

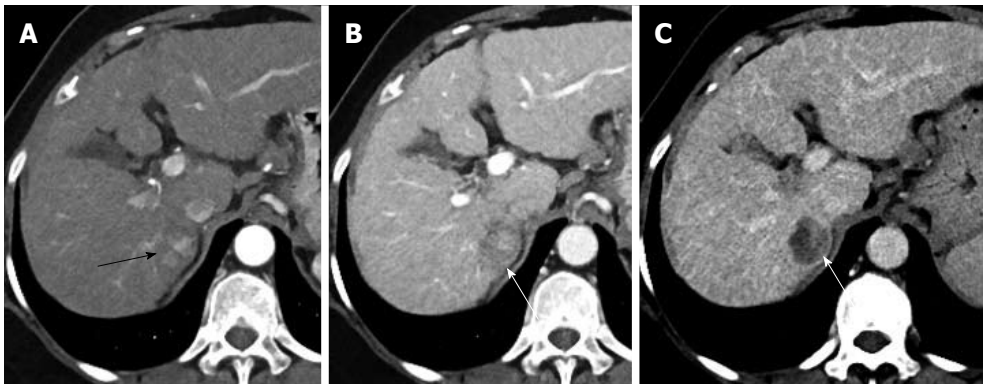

Figure 9 Cirrhotic liver with an arterially-enhancing lesion (black arrow) in segment 6 (A), which demonstrates a thin pseudocapsule (white arrow) in the portal venous (B) and delayed phases (C), better appreciated in the latter.

Pseudocapsule

This refers to a rim of peripheral enhancement in the portal venous or delayed phases (Figure 9). This sign may be well depicted in both CT and MRI and has been shown to be a significant predictor in the diagnosis of HCC[42,49]. A pseudocapsule has been found in 10%-47% of cases depending on the series studied[57-59].

Vascular invasion

Portal vein tumour thrombus (PVTT) is a well-known complication of HCCs; such invasion helps distinguish HCC from secondary hepatic cancers which rarely invade intrahepatic vessels[60]. It is important to note that the presence of a tumour thrombus can modify typical imaging features of HCC. When HCC infiltrates a portal vein, it continues to receive arterial blood supply and the tumor may drain directly into the portal vein. This direct draining results in arterioportal shunting and changes in portal vein haemodynamics[61]. Large HCCs complicated by PVTT less often demonstrate typical arterial enhancement with subsequent washout. Instead, the PVTT itself can show arterial phase enhancement with subsequent washout with distension of the vein (Figure 10)[61]. This arterioportal shunting may also result in poor enhancement of the surrounding liver parenchyma.

Lack of iron content

Presence of iron is better appreciated on MRI as opposed to CT and is shown as marked hypointensity on T2W sequences. Iron is normally present in the Kupffer cells that reside in sinusoids and are abundant in normal liver parenchyma. The presence of iron is highly suggestive of a non-malignant lesion in a cirrhotic liver[48]. On the contrary, the presence of an iron-free lesion in an otherwise iron-laden liver may suggest HCC (Figure 11).

Nodule-in-nodule appearance

This refers to the presence of a nodule within a larger nodule and is usually the result of the development of HCC within a pre-existing cirrhosis-related nodule. The nodule within the larger lesion may demonstrate increased arterial enhancement or T2 signal intensity relative to the surrounding larger nodule (Figure 12).

USE OF HEPATOBILIARY CONTRAST AGENTS

Hepatobiliary MRI contrast agents are increasingly being used and gradually changing the standard of diagnosis of HCC. These agents are gadolinium chelate-based with an initial vascular phase that is

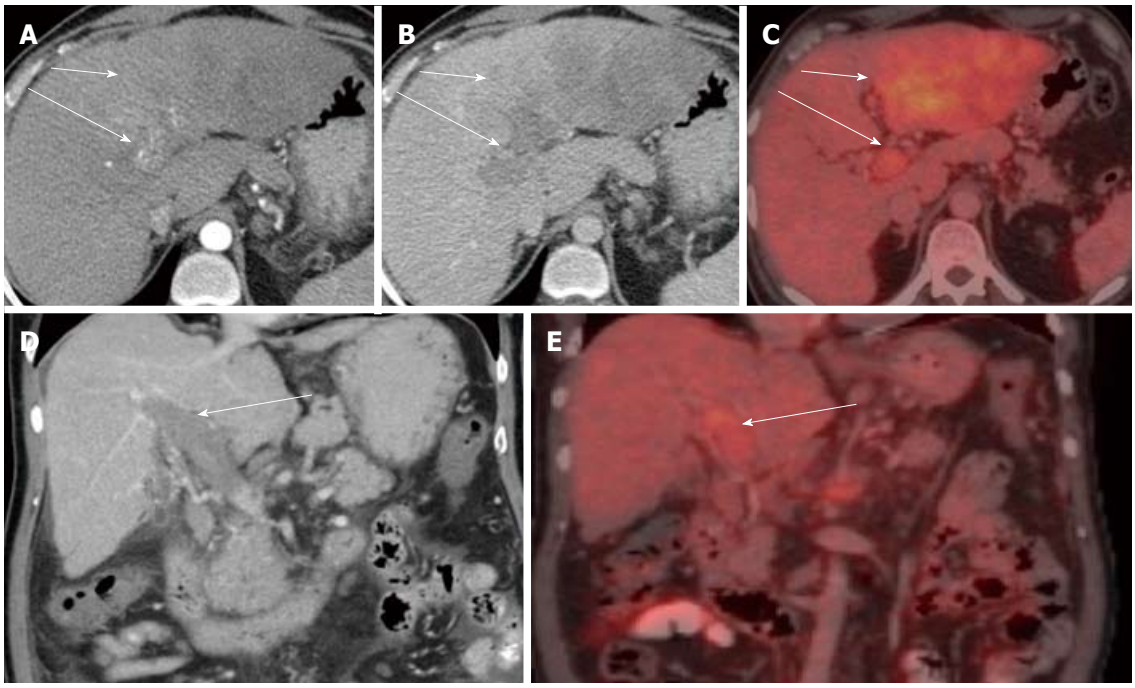

Figure 10 Vascular invasion. A large ill-defined left lobe mass with no significant arterial enhancement (A) and washout in the portal venous phase (B). An FDG-PET CT was done which revealed uptake in the left lobe mass (C) consistent with a hypermetabolic tumour. Arterial enhancement is noted within the distended thrombus filled portal veins in (A) with subsequent washout (B) suggestive of tumour thrombus. The tumour thrombus also demonstrates increased uptake on FDG-PET (C). Coronal images better depict the distended thrombus filled portal vein (D) with increased uptake on PET/CT (E) (short arrow: tumour; long arrow: tumour thrombus). PET: Positron emission tomography; CT: Computed tomography; FDG: Fluoro-2-deoxy-D-glucose.

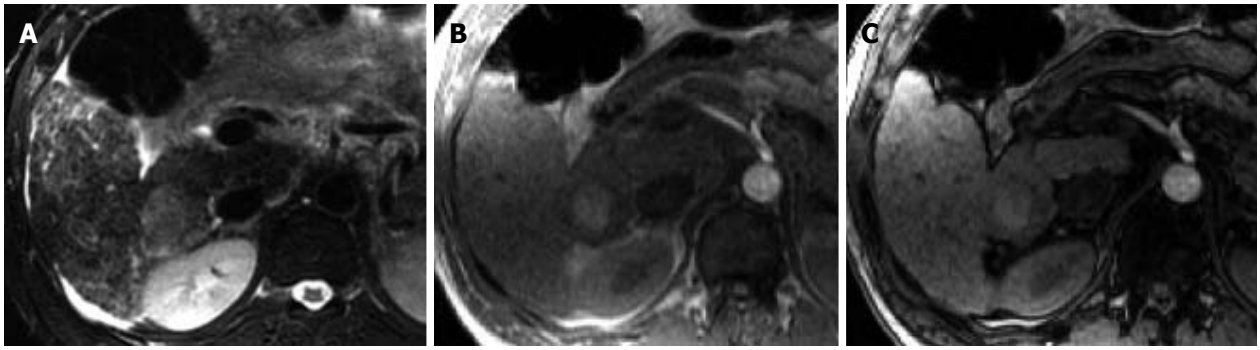

Figure 11 An iron-laden liver in a patient with hemochromatosis demonstrates a T2W hyperintense lesion (A) which is iron-free in the in- (B) and opposed (C) phases suggestive of hepatocellular carcinoma.

similar to the extracellular agents. However, they are actively taken up by hepatocytes *via* a group of proteins expressed in hepatocytes along the sinusoidal membrane known as organic anionic transporting polypeptides (OATP)[16]. In humans, OATP 8 appears to be responsible for cellular uptake[62]. The contrast agents are then partially excreted into the biliary system. Two hepatobiliary MRI contrast agents are currently in use: gadobenate dimeglumine (Gd-BOPTA, Multihance, Bracco, Milan, Italy) and gadoxetate dimeglumine (Gd-EOB-DTPA, Primovist in Europe and Eovist in the United States, Bayer Healthcare). Both contrast agents can be injected as an intravenous bolus dose. The hepatobiliary phase is attained 1-3 h after injection of Gd-BOPTA and about 20 min after injection of Gd-EOB-DTPA. With Gd-BOPTA, only 5% of the drug is transported through hepatocytes and excreted into bile whereas with Gd-EOB-DTPA, approximately 50% of the drug undergoes biliary excretion.

A small dose of Gd-EOB-DTPA (0.025 mmol/kg) is required compared to 0.1 mmol/kg for Gd-BOPTA. The former therefore has significant advantages in terms of safety, timing of examination and potentially better contrast. However, due to the low volume injected with Gd-EOB-DTPA compared to Gd-BOPTA, the vascular phase images are less ideal with a narrower imaging window for late hepatic arterial phase acquisition[63] which is when peak arterial enhancement of a nodule typically occurs. This can be overcome by performing multiple acquisitions during the arterial phase. Gd-EOB-DTPA does not provide a conventional delayed phase

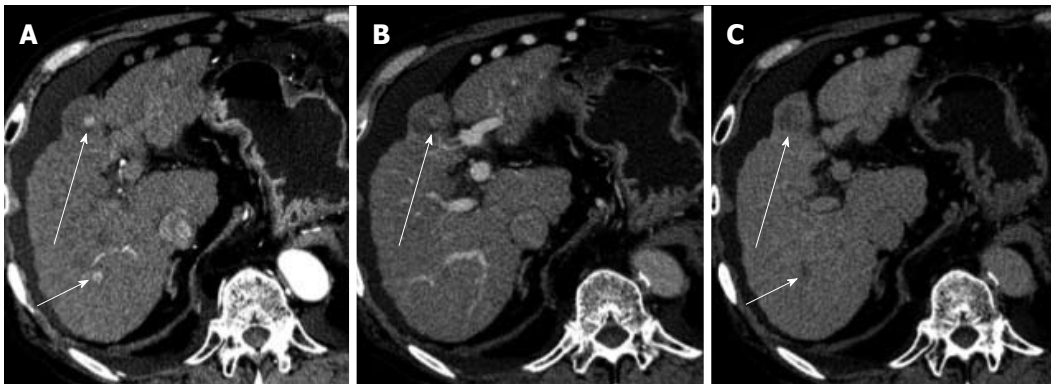

Figure 12 A focus of arterial enhancement is noted within a larger hypodense nodule (A) which demonstrates washout in the portal venous (B) and delayed (C) phases suggestive of development of hepatocellular carcinoma within a pre-existing cirrhosis-related nodule (long arrow). Another focus of hepatocellular carcinoma (short arrow) is noted more posteriorly demonstrating arterial enhancement (A) and delayed phase wash-out (C).

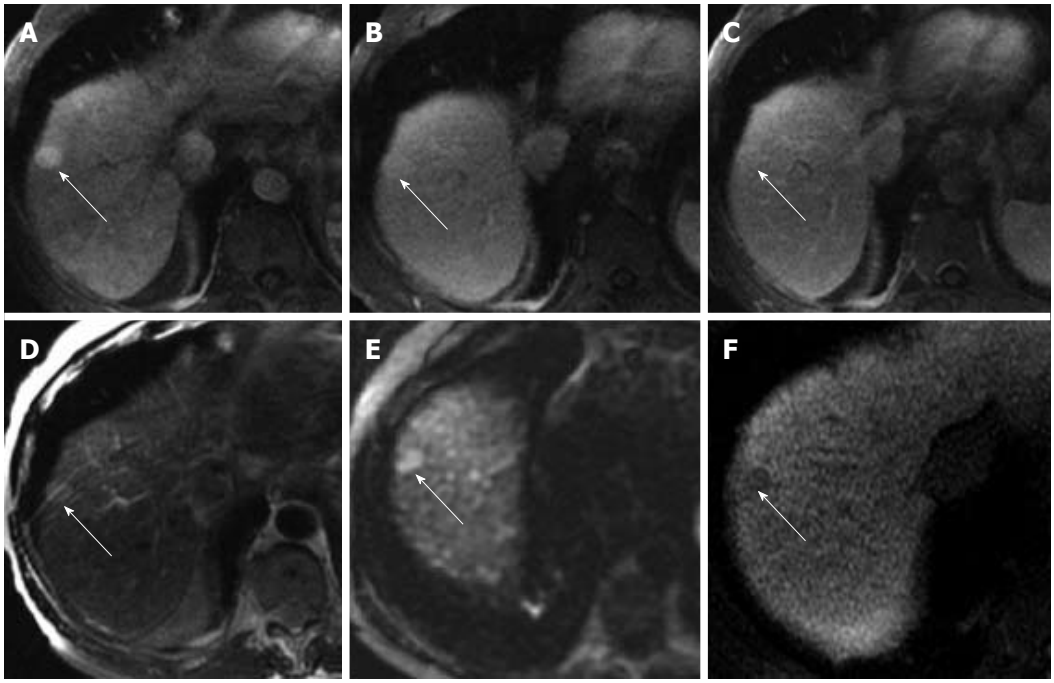

Figure 13 An initial study was performed using Gd-DTPA. This showed an arterially-enhancing lesion (A) with no evidence of wash-out or pseudocapsule on the portal venous (B) or delayed (C) phases with hyperintensity on T2W (D) and DWI (E) sequences. A follow-up study acquired two months later with Gd-EOB-DTPA demonstrated a hypointense lesion on the hepatobiliary phase (F), increasing diagnostic confidence of hepatocellular carcinoma.

as hepatocellular uptake occurs during its first pass through the hepatic sinusoids[64]. Hence, by the end of the portal venous phase, considerable hepatocellular uptake has occurred with both intracellular and extracellular pools of Gd-EOB-DTPA contributing substantially to parenchymal enhancement[64]. As this phase represents a transition from extracellular-dominant to intracellular-dominant enhancement, it may be termed the transitional phase[65].

In addition to increased cellularity and neovascularity in the multistep carcinogenesis of HCC, OATP expression gradually decreases in the development of HCC. This results in a lack of uptake in the hepatobiliary phase; most HCCs are hypointense in the hepatobiliary phase (Figure 7) whereas most non-HCC cirrhosis-associated nodules are iso- or hyperintense secondary to preservation of OATP 8 expression[66]. It is however important to note that 5%-10% of HCCs are iso- or hyperintense to liver in the hepatobiliary phase[67,68]. The addition of hepatobiliary phase sequences improves sensitivity of diagnosis of HCC by 5%-15% with Gd-EOB-DTPA (Figure 13)[69,70] and around 10% with Gd-BOPTA[71]. Interestingly, a study showed that 96% of HCC lacking arterial enhancement with subsequent washout (seen primarily in early HCCs) were hypointense during the hepatobiliary phase[72]. This is likely the most significant benefit of the use of hepatobiliary contrast agents in determination of HCC. It is important to note however that all non-hepatocellular lesions appear hypointense

on the hepatobiliary phase. Hence, it is imperative to interpret this phase in conjunction with that of the other sequences.

SUPPLEMENTARY IMAGING TECHNIQUES

Utility of non-contrast enhanced phase

Addition of a non-contrast enhanced phase (NC-CT) to a multi-phase CT study has been found to useful in providing a baseline for assessment of arterial phase enhancement and improving diagnosis of HCC[73]. The current practice of characterizing enhancement and washout with dynamic CT is performed qualitatively by visual assessment of the lesion relative to the surrounding liver parenchyma. This assessment is thus dependent upon variables that can influence liver attenuation such as steatosis and iron deposition. With the addition of NC-CT, even if a lesion was found to be isodense on the arterial phase, the observation of hypodensity on NC-CT would imply hypervascularity of the lesion.

Perfusion imaging

The improved temporal resolution of newer and faster multidetector CT systems allows perfusion studies of the liver[74]. CT perfusion is a method to analyze hemodynamic changes in tissue; it allows for quantitative assessment of various parameters such as tumour blood flow, blood volume, mean transit time and permeability-surface area product[75]. The liver has dual blood supply and neoarterialization occurs with the development of HCC resulting in alteration of perfusion parameters. Blood flow, blood volume, arterial perfusion and hepatic perfusion index were found to be significantly higher in HCC relative to hepatic parenchyma[76,77]. Sahani *et al*[75] also found that mean blood flow, blood volume and permeability-surface area product were higher in well-differentiated HCC than in moderately or poorly differentiated tumours. Additionally, it has been suggested that perfusion parameters can be utilized as biomakers to monitor treatment response in tumours[78].

Dual-energy CT

Conventional MDCT uses a polychromatic X-ray spectrum provided by a single X-ray tube whereas dual-energy CT (DECT) uses two different energy spectra produced by two different kVp settings. This is achieved by using two X-ray tubes at different tube currents with two corresponding detectors or with a single source X-ray tube with fast peak kVp switching. It is based on the premise that tissues demonstrate different attenuation at different energy levels. This allows for enhanced tissue differentiation and characterization, reduction of artifacts, iodine conspicuity and improvement of contrast-to-noise ratio (CNR) and signal-to-noise ratio (SNR)[79]. The attenuation of a material increases as its photon energy decreases. Materials with higher atomic numbers, such as iodine, portray a much greater attenuation increase as the photon energy decreases. This provides the basis for greater attenuation separation between tumour and liver parenchyma[80]. Gao *et al*[81] found that monochromatic images obtained using single source DECT can enhance the CT attenuation of iodine contrast media at lower energy levels in the enhanced arterial phase which aids in the identification of more and smaller HCC lesions (Figure 14). DECT may also improve detection of fat within hepatic lesions which may be indicative of HCC[82].

MR elastography

MR elastography (MRE) is a technique which is used for quantitative assessment of tissue stiffness and its most common clinical application is for evaluation of liver stiffness in the diagnosis of hepatic fibrosis[83]. In this technique, hepatic stiffness is measured using low-frequency mechanical shear waves generated by a source that is propagated through the liver. Liver stiffness increases systematically with stage of fibrosis; using a shear stiffness cut-off value of 2.93 kPa, the predicted sensitivity and specificity for detecting all grades of liver fibrosis is 98% and 99% respectively[84]. Malignant tumours have greater stiffness values than benign tumours and normal liver parenchyma[85-87]. Hence, MRE has shown to be a promising non-invasive tool for the imaging and characterization of solid hepatic tumours (Figure 15). A threshold value of approximately 5.0 kPa may be useful for differentiating benign focal lesions from malignant tumours[85]. The utility of MRE for differentiation of malignant tumors of liver is not well established and still under research.

MR spectroscopy

MR spectroscopy (MRS) allows for the non-invasive interrogation of the presence and concentration of various metabolites in tissue and hence aid in the provision of information with regards to tumour pathophysiology and metabolism[88]. It utilizes the magnetic properties of certain atomic nuclei; the more common ones employed are proton (^{1}H), phosphorus-31 (^{31}P) and carbon-13 (^{13}C). ^{1}H is the most commonly studied as it has the highest sensitivity. In liver tumour studies, the lactate resonance is related to energy metabolism of the tumour. Proton resonances of mobile lipids and the peak of total choline have been investigated as biomarkers to identify malignant tumours[88]. An increase in phosphomonoesters is associated with liver tumour progression and successful treatment is associated with a reduction of these levels[89-91]. Hence, ^{31}P MRS can potentially be used for treatment monitoring. MRS with ^{13}C has barely been utilized to examine human liver metabolism due to its technical complexity and relatively low sensitivity[88]. However, new techniques such as hyperpolarization

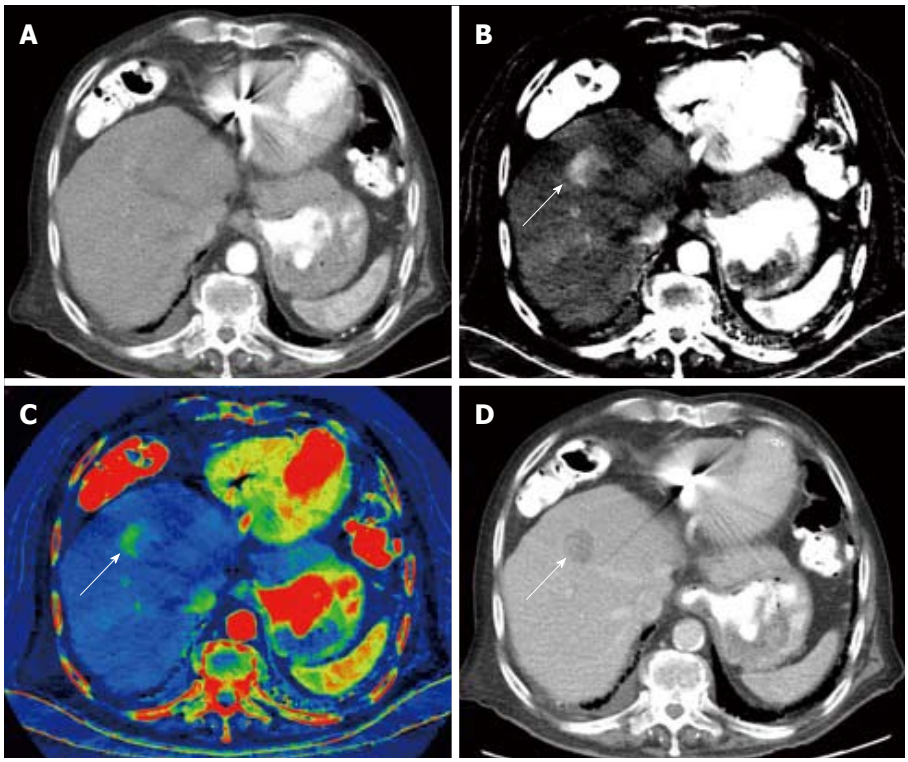

Figure 14 Dual-energy computed tomography of hepatocellular carcinoma. Nodular outline is suggestive of cirrhosis, arterial phase single energy computed tomography (SECT) image acquired at 140kVp demonstrates a vague focus of arterial enhancement that is difficult to differentiate from surrounding liver parenchyma (A), arterial phase DECT material decomposition iodine (MD-I) image shows uptake of iodine independently from inherent tissue attenuation, clearly demonstrating a nodular hyperenhancing lesion (B), arterial phase color overlay MD-I image also depicts the lesion well (C). Portal venous phase SECT image acquired at 120 kVp demonstrates characteristic wash-out (D). MD-I images improve detection and characterization of this small hepatocellular carcinoma. (Courtesy of Drs. Andrea Prochowski and Dushyant Sahani, Massachusetts General Hospital, Boston, MA, United States).

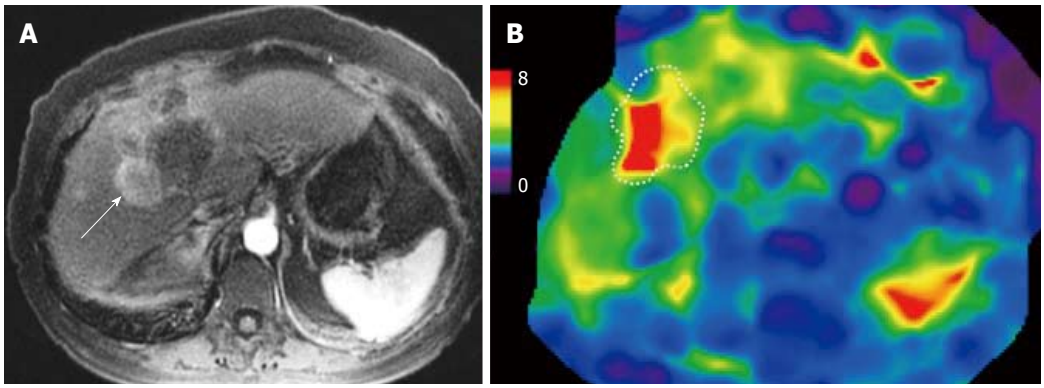

Figure 15 Magnetic resonance elastography of hepatocellular carcinoma. Arterial phase image (A) and stiffness map (B) from magnetic resonance elastography. The color scale of the stiffness map is expressed in kilopascal (kPa). A case of chronic alcoholic liver disease with liver stiffness of 5.3 kPa consistent with cirrhosis. The enhancing hepatocellular carcinoma (white arrow) has mean stiffness of 8.2 kPa suspicious for a malignant tumour. Note the tumor is stiffer in the more hyper enhancing regions of the tumour.

of ^{13}C-labeled glutamine has shown potential in the detection of small HCC in a cirrhotic liver[92].

Intravoxel incoherent motion imaging

DWI is a technique used for imaging molecular movement or diffusion. ADC in conventional DWI is influenced by two types of molecular movement: molecular diffusion and microcirculation in vessels (perfusion-related diffusion)[93]. With high *b*-values, the effect of perfusion-related diffusivity is largely eliminated and the ADC value can estimate true molecular diffusion. The effect of perfusion-related diffusion, however, cannot be completely removed. Hence, DWI performed using a range of low and high *b*-values or intravoxel incoherent motion imaging (IVIM) imaging has been employed to measure diffusion and perfusion-related diffusion separately[94,95]. Post processing of IVIM sequences can generate several

parameters including: the D value (true diffusion that reflects intra- and intercellular molecular movement) and the pseudo-diffusion coefficient D* which reflects the microcirculation in the vessels or perfusion-related diffusion; perfusion fraction (Pf) and ADC. D and D* aspects can be separated using biexponential fitting of the DWI data[95]. It is well-established that ADC values of malignant hepatic lesions are lower than that of benign lesions[96,97]. However, measured ADC values show substantial variability secondary to differences in choice of *b*-values[98]. Diffusivity values acquired using the IVIM model, however, are less influenced by the choice of *b*-values and may provide consistent and reproducible results[99]. Ichikawa *et al*[99] found that both the D and D* value of malignant hepatic lesions was suppressed compared with that of benign lesions and that the D value was a more reliable parameter between the two. IVIM-derived D values have been found to show significantly higher accuracy compared with ADC in differentiating high- from low-grade HCC[100]. Additionally, since D* reflects microcirculation, it may be possible to assess the effect of antiangiogenic drugs in HCC[101]. The early results show promise of IVIM in differentiating HCCs from benign nodules, however evidence for clinical utility is still lacking.

2-(^{18}F)fluoro-2-deoxy-D-galactose PET/CT

Positron emission tomography (PET) with the glucose analogue 2-(^{18}F)fluoro-2-deoxy-D-glucose (FDG) is extensively used in oncologic imaging. FDG-PET may be able to demonstrate increased uptake with HCC (Figure 10), however, it may miss 30%-50% of HCC lesions as the uptake is similar to the uptake in surrounding liver parenchyma[102-104]. Fluro-2-deoxy-D-Galagctose (FDGal) is touted as a hepatocyte-specific PET tracer for HCC; it is a tracer for galactose metabolism and avidly accumulates in the liver compared to other tissues[105]. It has potential not only as a PET tracer for detection of extra- but also intra-hepatic HCC. Sørensen *et al*[106] presented the first clinical study on the potential use of FDGal PET/CT for the detection of HCC and found high specificity in a retrospective study. Detection of HCC were comparable to that of multiphase contrast-enhanced CT. Additionally, FDGal PET/CT detected more nodules than other imaging modalities at the time of investigation and follow-up revealed rapid progression in those lesions. This may indicate the ability of FDGal to detect more lesions at earlier time points than conventional morphology based imaging modalities.

CONCLUSION

Imaging plays an imperative role in the diagnosis of HCC. The hallmark feature of arterial enhancement followed by washout is highly specific in at-risk patients and forms the foundation of current diagnostic guidelines. Difficulties in accurate diagnosis are largely secondary to lesions of small size. Ancillary features can aid in diagnosis and its use has been incorporated into Li-RADS. The use of hepatobiliary contrast agents has shown great promise in several studies with the ability to identify high grade dysplastic nodules and early HCC prior to neo-arterialization and progression to overt HCC, it may well be endorsed in future guidelines. Several other imaging techniques have also been investigated, many of which show potential that may shift the paradigm of HCC imaging assessment in the future.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Molecular imaging and therapy targeting copper metabolism in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Significant efforts have been devoted to identify new biomarkers for molecular imaging and targeted therapy of HCC. Copper is a nutritional metal required for the function of numerous enzymatic molecules in the metabolic pathways of human cells. Emerging evidence suggests that copper plays a role in cell proliferation and angiogenesis. Increased accumulation of copper ions was detected in tissue samples of HCC and many other cancers in humans. Altered copper metabolism is a new biomarker for molecular cancer imaging with position emission tomography (PET) using radioactive copper as a tracer. It has been reported that extrahepatic mouse hepatoma or HCC xenografts can be localized with PET using copper-64 chloride as a tracer, suggesting that copper metabolism is a new biomarker for the detection of HCC metastasis in areas of low physiological copper uptake. In addition to copper modulation therapy with copper chelators, short-interference RNA specific for human copper transporter 1 (hCtr1) may be used to suppress growth of HCC by blocking increased copper uptake mediated by hCtr1. Furthermore, altered copper metabolism is a promising target for radionuclide therapy of HCC using therapeutic copper radionuclides. Copper metabolism has potential as a new theranostic biomarker for molecular imaging as well as targeted therapy of HCC.

Key words: Hepatocellular carcinoma; Positron emission tomography; Copper metabolism; Radionuclide therapy; RNA interference; Gene therapy

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Core tip: Copper is required for cell proliferation and tumor angiogenesis. This article provided an up-to-date review of copper metabolism as a novel theranostic biomarker in hepatocellular carcinoma. Altered copper metabolism is not only a novel biomarker for molecular imaging of extrahepatic metastasis of hepatocellular carcinoma using radioactive copper, but is also a promising target for copper modulation and radionuclide therapy of hepatocellular carcinoma.

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INTRODUCTION

Copper is a trace metal that is required for numerous metabolically important enzymes involved in various metabolic pathways of human physiology[1,2]. These include ceruloplasmin, superoxide dismutase, dopamine monooxygenase, lysyl oxidase, cytochrome c oxidase, factor V, and tyrosinase. These enzymes are used for a variety of purposes such as melatonin production, bone production, thrombosis and neurotransmitter synthesis. The amount of daily dietary copper required for an average adult is 1.0 to 1.6 mg according to the third National Health and Nutrition Survey[3]. Zero point nine mg per day of copper is the recommended daily allowance, and less than 10 mg per day is recommended by the National Academy of Sciences[4]. The adult human body contains about 75 mg of copper[5]. The liver and brain contain about one third of the overall quantity present, but copper is distributed throughout the human body and found in many organ systems, including the heart, kidneys, pancreas, spleen, bone and muscle[5].

The majority of daily copper intake is from vegetables and legumes, with other sources such as various meats. On average, vegetable sources of dietary copper require a more complex enzymatic process for absorption, compared to non-vegetable sources such as meat or milk. The variable amount of copper in food sources is dependent on the various amounts of copper in the soil as well as different food processing techniques[1,6]. When copper is ingested *via* food sources, dietary absorption of copper predominantly occurs in the stomach and small bowel, with only approximately 30%-40% of ingested copper being absorbed by those living in industrialized countries. However, depending on dietary intake of copper, the human body can theoretically absorb as much as 63%-67% in a copper deficient diet, or as little as 12% in those whose copper intake is very high. The high acidic environment in the stomach is believed to cause the release of copper from natural organic complexes. Subsequently, absorption in the small bowel is influenced by a change in the pH as well as pancreatic enzymes[6-8].

Metallothionein within the absorptive cells of the bowel are able to bind copper *via* mercaptide bonds and then release it in the plasma cell membrane on the serosal side. After being released from the intestinal mucosa, copper is bound to amino acids and albumin in the portal venous system. A small portion of the copper in the portal venous system is able to pass through to the systemic circulation, while the remainder is transferred into the cytosol of hepatocytes *via* cell membrane receptors. Within the liver, copper is bound to various proteins, but preferentially metallothionein[5,9].

The liver is a critical organ in the systemic regulation of copper metabolism and the maintenance of copper homeostasis. Wilson's disease (WD) is an inherited copper metabolism disorder caused by mutation of the ATP7B gene located on chromosome 13, for which numerous specific mutations have been identified[10-12]. Long-Evans Cinnamon rat, an animal model of WD, has a deletion in the copper transporting ATPase gene and develops hereditary hepatitis followed by spontaneous hepatocellular carcinoma (HCC)[13]. When these rats are treated with the copper chelating agent D-pencillamine, as commonly used in humans with WD, prevention of the onset of hepatitis and the inhibition of elevated serum transaminases were observed[14]. Togashi *et al*[14] therefore concluded that abnormal copper accumulation in the liver of Long-Evans Cinnamon rats was associated with the pathogenesis of hereditary hepatitis and subsequent development of HCC. Both low and high molecular weight copper binding species have been described. The high molecular weight species predominate in gallbladder bile, while low molecular weight species are more prevalent in hepatic bile. The low molecular weight species are thought to assist in the membrane transport of copper across the biliary canaliculus. The high molecular weight portion of copper binding species is principally related to copper homeostasis[9,15]. This is supported by the inability to adequately remove hepatic copper in the absence of the higher molecular weight copper binding species, in the setting of protein synthesis inhibitors[16]. Copper that is tightly bound to bile salts is unable to be absorbed in the gastrointestinal tract, and is lost in feces, which is the predominant route of excretion[5,6,9,17].

The plasma concentration of copper has been shown to increase throughout life, peaking around the age of 60, and then having a minimal decline[18]. This process is thought to be related to a progressive reduction in biliary clearance later in life, rather than an

increase in gastrointestinal absorption[6,19]. Differences in the plasma concentration of copper have also been demonstrated due to gender, with females on average having higher concentrations than men. Women between the ages of 20 and 59 were shown to absorb more and have a quicker turnover of radiolabeled copper in a meal, when compared to men. Higher levels of ceruloplasmin are also present in females[18].

COPPER AND HCC

HCC is the fifth most common cancer worldwide. It is the third leading cause of cancer-related death worldwide. Overall, about 75%-80% of HCC occurs in patients with hepatitis B or C, with many other known risk factors including aflatoxin B1, obesity, alcohol usage, diabetes, and tobacco[20,21]. It was demonstrated that the copper content in hepatic parenchyma of patients with HCC was significantly higher than in those without HCC, with no significant difference in hepatic iron levels. In fact, the copper liver level was the only significant factor associated with the presence of HCC in the cohort of patients with hepatitis C and chronic liver disease[22]. There were reports of an increased incidence of HCC in patients diagnosed with WD[23,24]. The copper content and level of copper binding proteins in HCC has been shown to be higher than those seen in other liver malignancies such as cholangiocellular carcinoma and metastatic tumors[25,26]. In addition, the serum copper to zinc ratio was significantly higher in patients with HCC than matched controls[27].

There have also been reports of a decreased incidence of HCC in patients with copper metabolism disorders[28]. It has been proposed that WD patients treated with D-penicillamine have an elevated risk of developing HCC[29]. This may be secondary to the associated decrease in copper content in the liver, when on chelation therapy. This discrepancy could reflect either a carcinogenic or a protective role of copper in the pathogenesis of HCC, which remains to be elucidated in further studies.

COPPER METABOLISM AS A BIOMARKER FOR METABOLIC IMAGING OF HCC

Currently, the detection of liver masses is predominantly evaluated using anatomic imaging modalities[30], such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI). Molecular imaging is gaining momentum and is being used in various disease states[31]. Positron emitting fluorine-18-2-deoxy-gluocose (F-18 FDG) is a radioactive tracer used for the assessment of glucose metabolism in both benign and malignant tissues. After being delivered to the cells *via* blood flow, F-18 FDG is transported by GLUT transporters and then phosphorylated intracellularly. Typically, the FDG-6-phosphatase is trapped within the cells, unless there is a high level of phosphatase activity, as seen in the liver[32]. Secondary to the high level of phosphatase in the liver, the sensitivity for detecting well differentiated HCC is poor. However, there is usually high F-18 FDG uptake in moderately and poorly differentiated HCC. Positron emission tomography/computed tomography using F-18 FDG (F-18 FDG PET/CT) is also useful for the detection of recurrence and extrahepatic metastasis of HCC[33,34].

The sensitivity of FDG PET/CT in the detection of HCC was found to be approximately 50%, compared to the sensitivity of CT (75%)[35]. However, Wang *et al*[36] showed improved performance in the detection of HCC when an early dynamic F-18 FDG PET/CT was performed 240 s after tracer injection. Even better detection rates were obtained when early dynamic and conventional delayed whole body information was used in combination. The detection rates improved from 56.7% to 91.9% when using whole body delay versus the combination of early dynamic and whole body scans, respectively. In patients who were scheduled to undergo liver transplantation, F-18 FDG PET/CT was found to be useful for predicting microvascular invasion by HCC. The presence of microvascular invasion by HCC was predicted when the ratio of maximum standardized uptake value (SUV) of HCC to mean SUV of normal liver parenchyma was 1.2 or greater[37].

C-11 acetate, a tracer that evaluates free fatty acid synthesis, may have better sensitivity than that of F-18 FDG[38]. According to a study performed by Ho *et al*[39], the sensitivity of HCC detection in patients with less than 3 lesions was 87% for C-11 acetate and 47% for F-18 FDG. When this was correlated with histologic findings, it appears that well differentiated tumors were better detected by C-11 acetate, while the poorly differentiated tumors were better detected by F-18 FDG. None of the non-HCC tumors demonstrated abnormal C-11 acetate uptake. The use of dual phase C-11 acetate, using the change in uptake values in early and conventional imaging, correctly differentiated between small, 1-3 cm, well differentiated HCC from focal nodular hyperplasia and hemangiomas[40].

The tracer C-11 choline is used to evaluate the metabolism of phospholipids subsequently used as constituents of the cell membrane. F-18 FDG negative HCC showed elevated uptake of C-11 choline, which was predominantly seen in the moderately differentiated group[41]. F-18 fluorocholine has also been shown to perform better than F-18 FDG for well differentiated HCC, with a combination of both tracers appearing to be the best option[42]. Compared to a single modality, a combination of imaging modalities, including F-18 FDG PET, CT, MRI and ultrasound, currently has higher sensitivity, with minimal effects on specificity[38].

Continuous efforts are being made to develop new tracers for molecular imaging of HCC. Radioactive

copper has been used for the assessment of copper metabolism disorders in patients diagnosed with WD using nuclear imaging for at least 45 years[43-46]. When exploring copper metabolism as a biomarker for molecular imaging of HCC, Peng *et al*[47], for the first time, demonstrated that mouse extrahepatic hepatoma could be visualized by PET using copper-64 chloride ($^{64}CuCl_2$) as a tracer, based on increased copper uptake mediated by mouse copper transporter 1 (mCtr1). There was relatively less ^{64}Cu uptake in the hepatoma compared to the liver, which was thought to be related to several factors: less mCtr1 in the tumor relative to the liver, the possibility that endogenous mCtr1 may be less active on the tumor, other copper transporters in normal hepatocytes not expressed on the tumor, and more rapid efflux of copper in tumor cells than in normal hepatocytes[48]. More recently, human HCC xenografts in athymic mice were also visualized by PET after intravenous injection of $^{64}CuCl_2$ as a tracer[49]. There was abundant physiologic distribution of ^{64}Cu in the liver, which resulted in limited evaluation of primary HCC. Given the normal intense uptake of FDG by cortical brain tissue and low physiological cerebral uptake of ^{64}Cu[47-53], $^{64}CuCl_2$-PET is a promising technique for non-invasive assessment of intracranial and other extrahepatic metastasis of HCC located in areas with low physiological copper uptake (Figure 1). The prognosis for patients with intracranial HCC metastasis is poor as they are often resistant to systemic chemotherapy. The use of $^{64}CuCl_2$-PET/CT for early detection of HCC intracranial metastasis is significant for improving the prognosis of patients with metastatic HCC. On the other hand, $^{64}CuCl_2$-PET is expected to be useful for excluding extra-hepatic metastases in pre-transplant work up of patients who are considered candidates for liver transplantation. Positron emitting ^{64}Cu radionuclide has a half-life of 12.7 h, making it possible to ship it to an imaging facility distant from the production site of this radiotracer. Preclinical radiation dosimetry of $^{64}CuCl_2$ using the *Atp7b*$^{-/-}$ knockout mouse model of WD was comparable to that of F-18 FDG[50], supporting the use of $^{64}CuCl_2$ as a radiotracer for PET of HCC metastasis, with the exception of the metastatic lesions in the abdomen due to excreted ^{64}Cu in the intestinal tract.

TARGETING COPPER METABOLISM FOR THE TREATMENT OF HCC

Early detection and treatment are most critical for reducing mortality in patients with HCC[54,55]. The use of conventional transarterial chemoembolization (TACE) for the treatment of unresectable HCC has been found to improve the overall survival of patients compared to available supportive care[56]. The use of cisplatin or doxorubicin in a large review comparing chemoembolization showed a significant benefit compared to embolization alone[57]. A major limitation in the literature regarding TACE is the lack of consistent methods between various investigators[56]. The use of TACE with drug eluting beads (DEB-TACE), primarily using doxorubicin allows for slow drug release and lower levels of systemic chemotherapeutic agents when compared to TACE using lipiodol[58]. Although no survival benefit was shown, Malagari *et al*[59] were also able to show longer times to progression, less recurrence, and a better local response when using doxorubicin-eluting beads compared to bland embolization. Despite additional studies not showing a difference in radiographic response, survival or adverse events[60], Sieghart *et al*[56], still recommend that any future trials should include drug eluting bead TACE secondary to lower systemic levels of doxorubicin and then a possible reduction in drug-drug interactions.

The ability to bridge a patient to liver transplant has been achieved using several types of neo-adjuvant therapies including TACE, radiofrequency ablation, trans-arterial radioembolization (TARE), external beam radiotherapy and surgical resection. Bridging has been shown to decrease waiting list dropout, reduce HCC recurrence, and improve post-transplant survival with the goal of obtaining similar post-transplant outcomes to non-HCC patients[61].

Palliation for patients with end-stage or terminal HCC includes various options, with the primary goal of improving patient symptoms rather than definitive treatment[62]. Average survival for patients with end-stage or terminal HCC is 3-4 mo, and includes about 15%-20% of all HCC patients at presentation. The treatment options for end-stage disease are opiates, acetaminophen and corticosteroids[62]. HCC can be difficult to treat despite significant efforts devoted to the development of effective therapies for the treatment of this devastating disease[55]. Continuous efforts are being made to identify new targets for the treatment of HCC. Angiogenesis is an important pathway in tumor growth and copper is an important angiogenic factor for tumor growth[63]. Copper has been shown to be a cofactor in several mediators of angiogenesis including angiogenin, matrix metalloproteinase and fibroblast growth factor[64-66]. Moriguchi *et al*[67] demonstrated the antiangiogenic effects of the copper chelator, trientine dihydrochloride, on HCC in a rat model. Other groups have also shown that the copper chelator, pencillamine, together with diet modification can lower copper levels and microvascular density in cerebral rabbit models. Brem *et al*[68] also concluded that using pharmacologic withdrawal and dietary depletion of copper suppressed intracerebral tumor angiogenesis. However, prolonged anti-copper cancer therapy with copper chelators or long-term use of D-pencillamine for anti-inflammatory treatment in rheumatoid arthritis has been shown to cause toxicity such as bone marrow suppression, rash and neurologic symptoms[69,70]. Significant

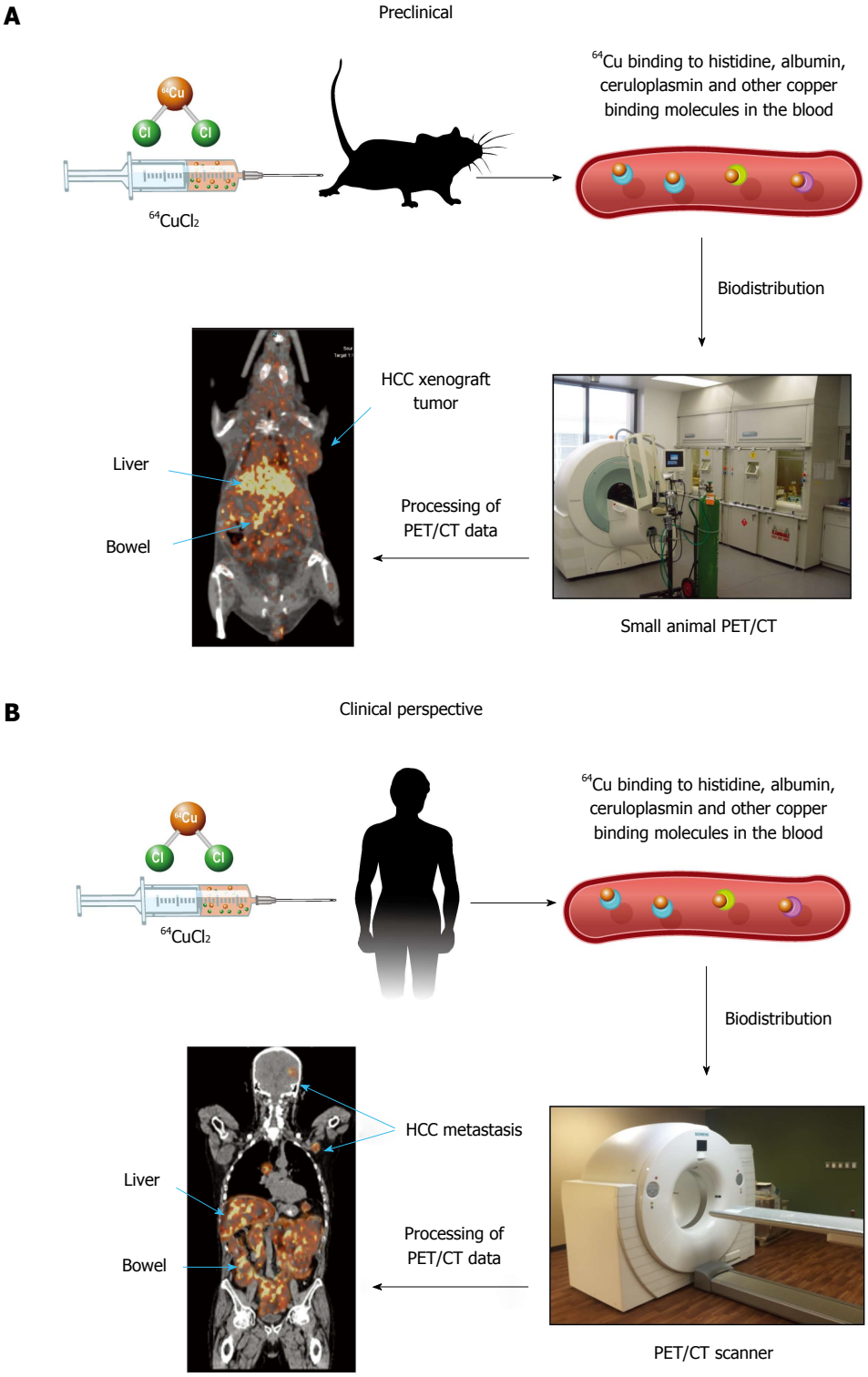

Figure 1 Metabolic imaging of metastasis of hepatocellular carcinoma with ^{64}CuCl$_2$-position emission tomography and computed tomography. A: Preclinical metabolic imaging of HCC xenografts in mice injected with ^{64}CuCl$_2$ as a tracer. ^{64}Cu bound to copper binding molecules in the blood immediately after intravenous injection of ^{64}CuCl$_2$. PET/CT images were then obtained that show the expected biodistribution of ^{64}CuCl$_2$ in the liver and intestinal tracts, with low uptake in the brain and muscle tissues. The HCC xenografts implanted on the shoulder showed increased ^{64}Cu uptake on PET/CT images; B: Schematic showing the clinical perspective of metabolic imaging of HCC in humans. Human patients may be injected with ^{64}CuCl$_2$ and subjected to PET/CT for detection of HCC metastasis in areas of low physiological ^{64}Cu uptake, such as brain and musculoskeletal tissues. HCC: Hepatocellular carcinoma; PET/CT: Hybrid positron emission tomography and computed tomography.

advancement has been made in understanding the molecular biology of copper transporters and chaperons regulating cellular copper homeostasis[71]. Recent advances in understanding the role of copper in the signal transduction pathway of cellular proliferation[53,72-76] support further study of copper metabolism as a target for molecular therapy of HCC. The selection of patients with copper hypermetabolic, metastatic HCC using $^{64}CuCl_2$-PET/CT may be helpful for improving the efficacy of anti-copper therapy of HCC. Human copper transporter 1 (hCtr1) is a high affinity copper transporter which mediates cellular copper uptake in humans[77]. To overcome the side effects of anti-copper therapy with long-term or high-dose use of copper chelators, RNAi-mediated knockdown of hCtr1[53] may be a promising approach for targeted anti-copper therapy of HCC.

The use of external beam radiation for HCC has been limited as the liver is considered a radiosensitive organ, which may have led to early under-dosing of patients[78]. This limitation can be compounded when HCC occurs in the setting of an already diseased liver as seen with hepatitis C. Radiation-induced liver disease in patients subjected to external beam radiation can cause endothelial damage, platelet activation, fibrin thrombus and venous occlusion. These changes can lead to subsequent hepatic fibrosis. However, there may be a role for radiotherapy in patients with tumors that are in challenging locations, for palliative purposes, a bridge to transplant, or in combination with other treatment options[79]. External beam radiation as well as percutaneous cementoplasty has been used for palliative purposes with successful management of symptoms[80,81].

The targeted delivery of radionuclide therapy has been carried out by intra-arterial delivery of various conjugates radiolabeled with therapeutic radioisotopes including yttrium-90, iodine-131, holmium-166 and rhenium-188[82,83]. Yttrium-90 labeled microspheres are used for interventional radionuclide therapy of HCC[84]. Currently, there are both glass- and resin-based particles available for radioembolization of HCC. The glass-based form has a smaller size with a reduced embolic effect and lower incidence of post-embolization syndrome. One limitation of TACE is possible decompensation of the liver after use in patients with hepatic artery and portal thrombus. The use of Y-90 glass microspheres in patients with HCC and branch or lobar portal vein thrombosis showed favorable tumor response rates and was safe in a trial which included 108 patients[85]. Y-90 does not emit gamma rays and is therefore not optimal for imaging. In contrast, Rhenium-188 is a therapeutic radionuclide with a physical half-life of 16.9 h and emits both beta and gamma rays. The use of intra-arterial Rhenium-188-conjugates for radioembolization of HCC has been shown to inhibit tumor growth[86]. Attempts were also made to develop I-131 radiogene therapy of HCC based on tumor-specific expression of the human sodium/iodide symporter (hNIS) under control of the alpha fetoprotein promoter and enhancer[87-89]. Tumor-specific expression of the hNIS in HCC cells was achieved by transfection of HCC cells with a vector encoding the hNIS gene driven by an alpha fetoprotein promoter/enhancer. Increased uptake of I-131 by the cells expressing hNIS was detected by gamma counting *in vitro* and by imaging with a gamma camera *in vivo*. Growth of extrahepatic tumor xenografts derived from cells expressing hNIS was inhibited, secondary to radiation effects of ^{131}I accumulated in the transfected HCC cells expressing hNIS[89]. The development of technologies to allow safe and efficient delivery of the vector encoding the hNIS gene is critical for the clinical application of I-131 radiogene therapy of HCC, based on the findings in preclinical studies.

Multiple copper isotopes are available for cancer imaging and therapy[90-93]. Copper-64 emits both β^+ and β^- particles and has potential as a theranostic copper radionuclide for both cancer imaging and therapy. Apelgot *et al*[94] demonstrated that ^{64}Cu had a lethal effect in mammalian cells similar to that of ^{67}Cu radionuclide. Significant efforts have been made to develop ^{64}Cu-radiolabeled conjugates for cancer imaging and therapy[95-99]. Based on its simplicity and increased tumor uptake of ^{64}Cu demonstrated by PET[47-49,52,53,100,101], ionic $^{64}CuCl_2$ has potential as a therapeutic radiopharmaceutical for the treatment of tumors expressing high levels of hCtr1. Recently, it was reported that growth of malignant melanoma overexpressing hCtr1 was suppressed in mice treated with $^{64}CuCl_2$[102]. In addition to its potential as a reporter gene for tracking gene delivery with PET, targeted overexpression of hCtr1 may be used for copper radiogene therapy of tumors expressing low levels of endogenous hCtr1[103]. The findings from preclinical studies support further investigation of ionic $^{64}CuCl_2$ as a radiopharmaceutical for targeted radionuclide therapy of HCC, in addition to copper modulation therapy with copper chelators (Figure 2).

CONCLUSION

Copper is a transitional metal required for the regulation of cell proliferation and angiogenesis. The exact role of copper in the development of HCC is still poorly understood, as demonstrated by the paradoxical suppression or increase of HCC in patients with copper metabolic disorders such as WD. The findings of increased uptake of radioactive copper by extrahepatic HCC xenografts in mice invite clinical exploration of altered copper metabolism as a new imaging biomarker for metabolic imaging of HCC metastasis with PET using $^{64}CuCl_2$ as a radioactive tracer. In addition, copper metabolism has potential as a target for copper modulation gene therapy of HCC

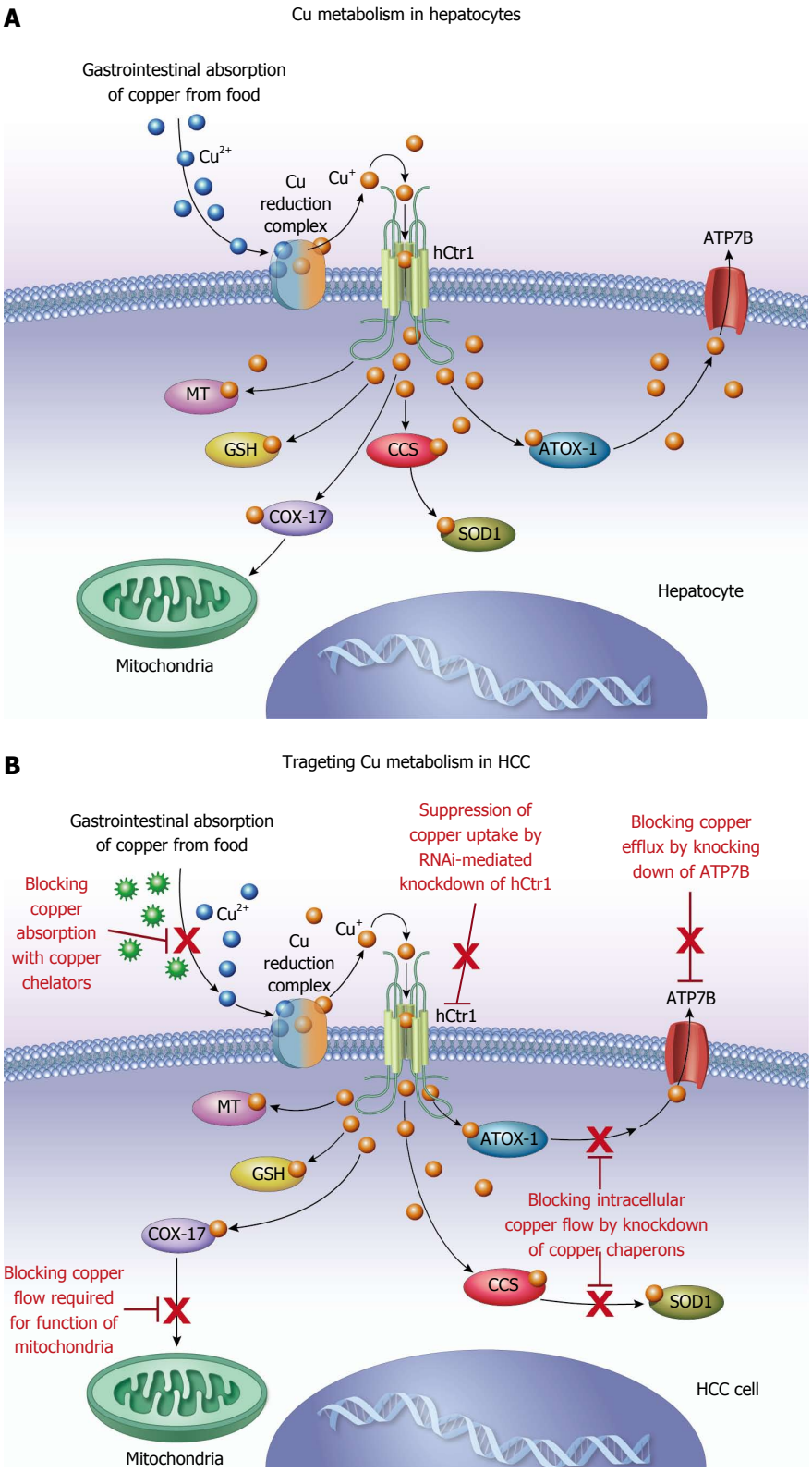

Figure 2 Perspective on targeting copper metabolism for the treatment of hepatocellular carcinoma. A: Copper metabolism in hepatocytes. Copper metabolism in hepatocytes is regulated by a network of copper transporters and chaperons. Following copper uptake mediated by the influx copper transporter, hCtr1, copper is transported intracellularly by copper chaperons and copper homeostasis is maintained by the outflow of copper mediated by the efflux copper transporter, ATP7B; B: Targeting copper metabolism for the treatment of hepatocellular carcinoma (HCC). Copper is required for cell proliferation and may play a role in the signaling transduction pathway regulating proliferation of HCC cells. Targeting copper metabolism with copper chelators has been tested for anti-copper therapy of HCC, with variable response. RNAi-mediated knockdown of hCtr1 and/or other copper chaperons is a potential new approach for targeted anti-copper gene therapy of HCC. Furthermore, ionic $^{64}CuCl_2$ or $^{67}CuCl_2$ have potential as new radiopharmaceuticals for systemic radionuclide therapy of HCC, based on increased ^{64}Cu uptake of HCC visualized on preclinical PET/CT images. hCtr1: Human copper transporter 1; ATOX-1: Antioxidant 1; Cox 17: Cytochrome c oxidase 17; CCS: Copper chaperone for superoxide dismutase; SOD1: Superoxide dismutase 1; GSH: Glutathione; MT: Metallothionein; ATP7A: Copper-transporting ATPase 1; ATP7B: Copper-transporting ATPase 2; PET/CT: Hybrid position emission tomography and computed tomography.

based on RNAi-mediated knockdown of hCtr1 followed by administration of copper chelators. Furthermore, $^{64}CuCl_2$ or $^{67}CuCl_2$ may be used as radiopharmaceuticals for radionuclide therapy of HCC and ablation of extrahepatic HCC metastasis.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Prediction of hepatocellular carcinoma biological behavior in patient selection for liver transplantation

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Abstract

Morphological criteria have always been considered the benchmark for selecting hepatocellular carcinoma (HCC) patients for liver transplantation (LT). These criteria, which are often inappropriate to express the tumor's biological behavior and aggressiveness, offer only a static view of the disease burden and are frequently unable to correctly stratify the tumor recurrence risk after LT. Alpha-fetoprotein (AFP) and its progression as well as AFP-mRNA, AFP-L3%, des-γ-carboxyprothrombin, inflammatory markers and other serological tests appear to be correlated with post-transplant outcomes. Several other markers for patient selection including functional imaging studies such as ^{18}F-FDG-PET imaging, histological evaluation of tumor grade, tissue-specific biomarkers, and molecular signatures have been outlined in the literature. HCC growth rate and response to pre-transplant therapies can further contribute to the transplant evaluation process of HCC patients. While AFP, its progression, and HCC response to pre-transplant therapy have already been used as a part of an integrated prognostic model for selecting patients, the utility of other markers in the transplant setting is still under investigation. This article intends to review the data in the literature concerning predictors that could be included in an integrated LT selection model and to evaluate the importance of biological aggressiveness in the evaluation process of these patients.

Key words: Hepatocellular carcinoma; Liver Alpha-fetoprotein; Transplantation; Biomarkers; Histopathology; Recurrence; Integrated prognostic tool

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Core tip: An integrated model predicting post-transplant survival of hepatocellular carcinoma patients after

liver transplantation has not yet been defined. Current selection criteria for liver transplantation that do not consider its biological aggressiveness are mainly based on morphological tumor markers that offer only a static view of the tumor. Many biomarkers predicting post-transplant outcome and stratifying those patients who are candidates for liver transplantation are under evaluation. An integrated prognostic model will make it possible to quantify the tumor burden *via* functional imaging modalities as well as biological markers.

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INTRODUCTION

Liver transplantation is the gold standard treatment for selected patients with hepatocellular carcinoma (HCC) as it cures both the tumor and the underlying liver cirrhosis. Since widespread use of liver transplantation (LT) is still limited due to organ shortage[1], reliable patient selection criteria are critical to maximize LT survival benefit. The equipoise between the impact that decision will have on the patients remaining on the waiting list and on the recipient him/herself must be based on reliable predictors of post-transplant outcome.

Explant pathology features constitute a direct expression of the tumor's biological aggressiveness and, in particular, of micromacrovascular invasion and dedifferentiated grading. The former, in particular, represents the most important marker of HCC aggressiveness[2-6], Iwatsuki *et al*[7] demonstrated a more than 4-fold increased risk of recurrence following transplant when microvascular invasion is detected. Although associated with an excellent ability to predict post-transplant HCC recurrence, microvascular invasion is difficult to detect at the pre-operative biopsy. Crucial information needed to predict the outcome and to guide the decision-making process about listings becomes available, therefore, only after the explant specimen has undergone pathologic examination. Although research has been attempting to identify new makers, gross morphology has until now been considered the primary prognostic parameter. Strict adherence to macromorphological criteria (*i.e.,* the Milan criteria) has been considered the best selection criteria for HCC patients in the transplant setting since the sizes and the number of the nodules are considered the best surrogates for microvascular invasion. Indeed, nodule size and number are the worldwide standard for patient selection in most centers. The Milan, the University of California, San Francisco (UCSF), the total tumor volume (TTV) and the Up-to-7, which[8,9] are associated with good overall middle to long term disease-free survival (DFS) rates after transplantation[10,11], are the most frequently used and scientifically validated morphology-based criteria.

It is widely recognized, nevertheless, that use of pre-transplant macromorphological criteria in the selection process of LT candidates poses a number of relevant drawbacks. It has likewise been shown that in a large proportion of patients (up to 15%-25%)[12]. There is a significant discrepancy between pre-transplant radiologic staging and explant pathology. These findings are partially attributable to the time-lapse between the last radiologic evaluation and the transplant itself during which the tumor and the staging may have progressed. The discrepancy may also be explained by inaccurate radiological data leading to an underestimation of nodule number/size. There could also be an incomplete overlapping of macromorphological traits and absence of microvascular invasion. In fact, although infrequent, small paucinodular HCC may present biologically aggressive features that would seem to predict an unfavorable post-transplant outcome. Finally, and more importantly, the strict adoption of macromorphologicical criteria could lead to the exclusion of a relevant number of patients who could, instead, benefit from transplant[13-17]. Despite being outside current transplant criteria, some multinodular HCCs and also, to a lesser extent, relatively large tumors, have been shown to possess a favorable biological behavior and an acceptable long term post-transplant DFS. In view of these findings, several clinical trials have focused on expanding the Milan criteria[9,18]. According to "the metroticket concept"[8], nevertheless, the further HCC staging criteria is expanded for LT, the greater the cost will be in terms of higher recurrence. Conversely, when morphological selection protocols such as the Milan one include other recurrence predictors, their performance in predicting tumor recurrence seems to be improved[19,20], thus suggesting that other prognostic factors do exist and may be useful in improving prognostic accuracy. These considerations underline how a better understanding of the proliferative activity of HCC tumors can help to improve the selection criteria for LT and optimize resource allocation.

Although a definitive characterization is at yet unattainable, much light has recently been shed on the assessment of HCC tumor aggressiveness; these data and our own findings are the object of the present article, which has been divided into five sections: (1) biohumoral markers; (2) radiologic features; (3) histology; (4) response to therapy; and (5) tumor doubling times.

BIOHUMORAL MARKERS

AFP

First described by Abelev *et al*[21] in 1963, alpha-fetoprotein (AFP) is a usually fetal-specific glycoprotein

Table 1 Hepatocellular carcinoma patients' pre-transplant α-fetoprotein level, outcome, and biological features according to some recent studies

Ref.	No. of patients	Static AFP cut-off (mg/L)	Dynamic AFP	Outcome for increasing AFP ranges	*P* value	Other biological features
Berry *et al*[31] (2013)	8659	≤ 15, 16-66, 66-320, > 320	-	6 yr OS: from 70% to 60%, 57%, 51%	-	-
Toso *et al*[26] (2011)	6478	≤ 100, 100-500, > 500	-	3 yr OS: from 71% to 60%, 51%	< 0.001	-
Mailey *et al*[32] (2011)	2253	≤ 20, > 400	-	4 yr OS: from 76% to 54%	< 0.001	-
Duvoux *et al*[20] (2012)	1033	≤ 100, 100-1000, > 1000	-	5 yr OS: from 68% to 51%, 39%	< 0.001	VI, poor differentiation
Todo *et al*[30] (2007)	653	≤ 200, > 1000	-	5 yr OS: from 73% to 34%	< 0.001	-
Fujiki *et al*[41] (2009)	144	≤ 200, > 800	-	5 yr RFS: from 90% to 40%	0.003	VI, poor differentiation
Sotiropoulos *et al*[12]	100	≤ 20, 20-200, 200-1000, > 1000	-	5 yr RFS: from 97% to 60%, 57%, 51%	0.0003	-
Hameed *et al*[45]	211	≤ 1000, > 1000	-	5- yr RFS: from 80.3% to 52.7%	0.025	VI
Kondili *et al*[46]	32	-	grate increasing, low increasing	In 5 Patents with recurrence AFP increased at a greater magnitude than in 27 without recurrence	-	-
Han *et al*[28]	48	-	≤ 50 mg/L per month, > 50 mg/L per month	1 yr RFS: from 90% to 40%	< 0.001	VI
Vibert *et al*[29]	153	-	≤ 15 mg/L per month, > 15 mg/L per month	5 yr RFS: from 76% to 54%	0.01	VI
Merani *et al*[47]	6817	-	Stable, ≥ 400, downstaged to < 400	ITT survival: from 81% to 48%	< 0.001	-

OS: Overall survival rate; RFS: Recurrence-free survival rate; ITT: Intention-to-treat; VI: Vascular invasion.

whose importance in the diagnosis of HCC is well established[22-25]. Increasing evidence[8,11,26-29] suggests, moreover, that it also has a role in predicting outcome after LT. Since many studies[30-33] have reported a correlation between pre-LT serum AFP and post-LT overall survival in patients with HCC, AFP is considered an independent predictor of post-transplant survival (Table 1). Todo *et al*[30] reported 1, 3, and 5-year survival rates, respectively, of 84%, 77%, 72% for AFP < 200 μg/L *vs* 65%, 42%, 34% for AFP > 1000 μg/L. Mailey *et al*[32] likewise showed that the 1, 3, and 5-year absolute survival rates of 92%, 82%, 74%, respectively, in patients whose AFP level was lower than 20 μg/L decreased to 82%, 63%, 52% among those with an AFP > 400 μg/L.

Berry *et al*[31] reported that transplant recipients with HCC and serum AFP levels ≤ 15 ng/mL at transplant did not have a higher post-transplant mortality (AHR = 1.03) with respect to those without HCC. Patients with 16 to 65 ng/mL (AHR = 1.38), 66 -320 ng/mL (AHR = 1.65), and > 320 ng/mL (AHR = 2.37) serum AFP levels had progressively worse post-transplant mortality rates in comparison with recipients without HCC. Those investigators also reported that patients outside the Milan criteria had excellent outcomes if their AFP levels were < 15 ng/mL, while those who fulfilled the Milan criteria but had high AFP serum levels had poor survival rates.

Other investigators have reported that HCC recurrence after LT was correlated to pre-transplant AFP levels[30,34-41]. Fujiki *et al*[41] demonstrated that the 1, 3, and 5-year recurrence-free survival (RFS) rates in 144 patients was, respectively, 97%, 91%, 90% when AFP was 200 μg/L. However when the AFP was higher than 800 μg/L the RFS were 65%, 40%, 40%. Evaluating 100 HCC transplant patients, Sotiropoulos *et al*[37] found 1, 3, and 5-year RFS rates were 100%, 97% and 97% for AFP < 20 μg/L *vs* 68%, 23% and 23%, respectively, for AFP values higher than 100 μg/L.

Biological behavior features such as vascular invasion and tumor grade[2-7,42] have also been shown to be correlated with AFP levels. Fujiki *et al*[41] demonstrated that AFP > 800 μg/L was associated with an increased risk of microvascular invasion and poor differentiation of HCC with respect to AFP < 200 μg/L. In addition, vascular invasion and tumor differentiation had the highest odds ratios (OS) with AFP levels in a multivariate analysis by Duvoux *et al*[20].

In view of this evidence, new transplant selection criteria that include AFP have been investigated. Carrying out a study on a population of 6478 patients, Toso *et al*[26] reported that both total TTV and AFP levels were significant predictors of survival. A combined patient selection score based on TTV and AFP was thus developed. Compared to all the other criteria systems tested, that score was found to be the best predictor of outcome (Table 2). Duvoux *et al*[20] subsequently studied a 2 cohort population (with training and validation groups) of patients who had undergone LT for HCC within the context of a multicentric retro-

Table 2 Integrated selection criteria schemes for liver transplantation in hepatocellular carcinoma patients

Ref.	Model	No. of Patients	Parameters	Cut-off (points)	Criteria	Endpoint	Criteria-in outcome	Criteria-out outcome	Validation
Toso *et al*[26]	TTV/AFP	6478	TTV AFP	⩽ 115 cm³, > 115 cm³ ⩽ 400 ng/mL, > 400 ng/mL	TTV ⩽ 115 cm³ AND AFP ⩽ 400 ng/mL	Corrected posttransplant 3-yr OS	> 65%	< 50%	Grat *et al*: 104 patients with similar results
Duvoux *et al*[20]	The AFP Model	Training cohort: 597 Validation cohort: 435	Longest Diameter No. of nodules AFP	< 3 cm (0), 3-6 cm (1), > 6 cm (4) 1-3 (0), ⩾ 4 (2) < 100 ng/mL (0), 100-1000 (2), > 1000 (3)	Sum of individual points ⩽ 2	posttransplant 5-yr RFS	7.7 % (Milan-in) 14.4% (Milan-out)	53.3 % (Milan-in) 47.6 % (Milan-out)	Notarpaolo *et al*[44]: 560 patients with similar results
Lai *et al*[211]	-	422	mRECIST AFP slope	Progression *vs* No progression ⩽ 15 ng/mL per mo, > 15 ng/mL per mo	No progression AND AFP solpe ⩽ 15	5-yr RFS 5-yr OS	RFS: 90% (Milan-in), 87% (Milan-out) OS: 88% (Milan-in), 83.5% (Milan-out)	RFS: 67.7% (Milan-in) 47% (Milan-out) OS 67.3% (Milan-in) 55.4% (Milan-out)	Not yet validated

AFP: Alpha-fetoprotein.

spective study and identified 3 independent pre-LT predictors of recurrence: the number of tumors, the tumor size, and the AFP level. These parameters were incorporated to develop a model stratifying low and high risk of recurrence. A 3-tier AFP level score was included in the model and cut-offs of 100 μg/L and 1000 μg/L were adopted to identify the 3 AFP groups. The model proved to have an impact on recurrence and on survival, and net reclassification improvement showed that its predictability was superior to the Milan criteria[20] (Table 2). Duvoux's AFP model is, in fact, currently used in France (www.agence-biomedecine.fr) and United Kingdom (www.odt.nhs.uk/pdf/advisory_group_papers/LAG/HCC_recommendations_IR_TS_b_NAS_Work _in_Progress.pdf 19); a value ⩽ 2 is used as inclusion criteria for LT in HCC patients. The model has recently received an external validation in Spain[43] as well as in Italy[44], and a recent United States study confirmed the strong prognostic power of an AFP > 1000 ng/mL threshold in HCC patients undergoing LT meeting the Milan Criteria[45].

Although static, and despite the fact that a clear, unanimous cut-off level has yet to be defined, it has been seen AFP does indeed predict outcomes of HCC patients undergoing LT. It is important to remember, however, that a single assessment of serum AFP levels are unable express dynamic changes in the tumor's biological behavior. Since tumor aggressiveness shows a tendency to progress, at least two time-spaced evaluations are needed to determine if the biological course is stationary or progressing. A study by Kondili *et al*[46] published in 2007 showed that a rapid increase in AFP levels before LT represents a risk factor for tumor recurrence. Another study by Han *et al*[28] demonstrated that AFP progression exceeding 50 μg/L per month was significantly correlated to both vascular invasion and poorly differentiated tumor grade. Vibert *et al*[29] who measured AFP levels once a month in 153 patients on waiting lists for LT with the intent of demonstrating the relevance of dynamic AFP variations, found that its progression was more predictive of tumor recurrence and poor survival after LT than any static value. A slope > 15 μg/L per month was identified as the cut-off value. As suggested by Merani *et al*[47] decreasing AFP values also seem to have a clinical significance. Studying 6817 HCC cases, they reported that patients successfully downstaged from AFP > 400 μg/L to AFP ⩽ 400 μg/L had better post-transplant outcomes than patients whose AFP remained > 400 μg/L after downstaging. In addition, both increasing and decreasing AFP levels were found to be relevant to the evaluation of the oncological behavior of HCC, identifying tumors tending toward either a positive or a negative evolution. In view of the biomarker's potential relevance, the investigators concluded that further studies are warranted to standardize the cut-off values and assessment time points.

Despite abundant data on AFP found in the literature, any conclusions for the time being can only be tentative in view of many unsolved issues. Firstly, since AFP is a biomarker, biases linked to different laboratory methods and processing techniques are unavoidable and comparisons of results from multiple laboratories/studies are uncertain. Secondly, it is probable that the frequent exclusion of LT patients who die within 30 post-operative days has restricted data regarding the most aggressive tumors. According to Hakeem *et al*[48], moreover, patients included in AFP studies are highly heterogeneous. Finally, prognostic evaluations based on AFP levels are made only with regard to patients whose serum levels are higher

than normal (> 20 μg/L)[49] despite the fact that a considerable percentage of HCC patients are AFP-negative. In a study by Yang *et al*[50] focusing on novel prognostic biomarkers for HCC, 48.3% of the 305 patients studied had AFP < 20 μg/L. Zhang *et al*[51] likewise reported that 30%-40% of HCC patients studied were AFP-negative. Although a dynamic evaluation of the biomarker (AFP slope) could partially obviate this problem, further studies specifically addressing HCC patients with in-range AFP values are warranted.

Des-γ-carboxyprothrombin

Des-γ carboxyprothrombin (DCP), also known as protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ), was described more than twenty years after the first description of AFP[52]. The role of DCP as a biomarker for the diagnosis of HCC has been confirmed over the year[53-56], just as has been its potential to detect HCC early, given the highly sensitive immune assay that has been developed[57-59]. DCP's sensitivity and specificity in diagnosing HCC appear to be better than those of AFP[60-62], and simultaneous testing of both markers has been proposed for tumor detection[63].

Interestingly, DCP has also been shown to be predictive of outcomes regardless of treatment[62,64-67]. Encouraging predictive values were first reported after ablative therapies for HCC[64,65,68]. According to Imamura *et al*[66] DCP was able to predict recurrences after resection for small HCCs and, similarly, Sakaguchi *et al*[67] demonstrated that DCP > 100 mAU/mL was associated to a negative prognosis in HCC patients within the Milan criteria undergoing resection. Some studies have shown that DCP has a predictive significance also with regard to LT outcome and that it is a powerful predictive serum marker. Basing their data on a cohort of 124 patients undergoing living donor liver transplantation (LDLT), Shindoh *et al*[69] found that the prognosis of these patients strongly depended on maximum pre-LT AFP or DCP values. Multivariate analysis performed on 144 HCC patients who underwent LDLT at the Kyoto University showed that DCP > 400 mAU/mL was an independent risk factor for tumor recurrence after transplant.

Fujiki *et al*[41] subsequently published the Kyoto expanded criteria for LDLT which included preoperative DCP levels < 400 mAU/mL, tumor size, and number[41]. A similar proposal was made by Taketomi *et al*[70] who suggested a different cut-off value for DCP (DCP < 300 mAU/mL). The role of DCP was recently confirmed in a United States population. A serum DCP ⩾ 7.5 mAU/mL in 127 HCC patients undergoing LT significantly correlated with tumor recurrence (HR = 3.5; 1.9-6.7). The HR increased when DCP was combined with AFP and the Milan criteria[71]. In addition, finding DCP expression in the liver of HCC patients, especially in the peritumoral tissue, both Inagaki *et al*[72] and Tang *et al*[73,74] suggested that a combination of serum and tissue DCP expression be utilized.

DCP's prognostic role seems to be linked to its association with elevated cellular proliferation and tumor growth rates[64,75] as well as high infiltrative growth and vascular invasion values[62,72,73,76]. Recently, Poté *et al*[62] reported that a serum level of DCP > 90 mAU/mL was an independent predictor of vascular invasion, while high DCP tissue expression was associated with poor tumor differentiation. *In vitro* studies have proven that PIVKA-Ⅱ is able to promote cellular proliferation and migration[77,78] just as it induces expression of angiogenetic factors such as endothelial grow factor receptor (EGFR) and vascular endothelial grow factor (VEGF)[79,80].

While both clinical and biological reports and *in vitro* studies support the view that DCP is an index of HCC aggressiveness, its relative clinical relevance is still under debate.

AFP mRNA

Post-transplant tumor recurrences are almost certainly due to residual cancer cells from the removed tumor, and detection of HCC cells in the peripheral blood seems to be a direct and accurate method to predict tumor recurrence[81]. At the same time, AFP mRNA expression in the peripheral blood is a reliable marker of circulating cancer cells[82] .

In 2005 our research group reported that the pre-operative AFP mRNA level is a significant predictor of survival after radical therapy for HCC[83]. Marubashi *et al*[84] likewise reported that a positive pre-operative test for peripheral blood AFP mRNA was found to be an independent risk factor for the recurrence of HCC after LDLT.

Using a nested-polymerase chain reaction (PCR) technique, Ijichi *et al*[85] reported, instead, that the pre-operative presence of AFP mRNA-expressing cells in the peripheral blood was not associated with after resection HCC recurrence.

Data supporting AFP mRNA's role as a predictor of HCC recurrence are as yet inconclusive. Toso *et al*[86] hypothesized that strategies to decrease the engraftment of circulating tumor cells could lower the risk of recurrence. Some of the strategies they proposed were selecting recipients with low baseline circulating HCC cells by adding biological markers to the accepted combination of morphological criteria and decreasing the perioperative release of HCC cells *via* careful perioperative handling of the tumor.

AFP-L3%

Given its high specificity and sensitivity in detecting tumors, in the early 90s some investigators began to consider lens culinaris agglutinin-reactive fraction of α-fetoprotein (AFP-L3%), the percentage of a fucosylated form of AFP over the total AFP level, an adjunctive marker for HCC diagnosis of[87-90].

The biomarker also seems to be able to predict prognosis. High AFP-L3% levels have, in fact, been reported to be correlated with poor outcome after transcatheter arterial chemoembolization (TACE)[91] and with a higher risk of recurrence after local ablation[92,93] and hepatectomy[92,94]. According to Kobayashi *et al*[95] AFP-L3% values are useful in predicting the outcome of patients with normal serum AFP levels[96]. Considering it a potential new generation tumor marker, Kusaba demonstrated that liver cancer cells expressing AFP-L3% showed a tendency towards early vascular invasion and intra-hepatic metastasis, staining more positive with Ki67and less with alpha-catenin[97]. Chaiteerakij *et al*[71] found that AFP-L3% was significantly associated with tumor recurrence in a population of 127 HCC patients undergoing transplantation. Interestingly, the HR increased from 2.6 (1.2-6.6) to 4.5 (1.9-10.6) when that parameter was added to the Milan criteria. A prognostic value was also attributed by that same study to the absolute AFP-L3% value.

There is still little evidence, nevertheless, to support the prognostic relevance of AFP-L3% in the LT context.

Other biomarkers: Many other HCC biomarkers after liver resection, loco-regional treatments or LT have been cited in medical literature but their potential prognostic role in the transplant population has yet to be well defined.

Furthermore, the systematic citation of each individual marker is beyond the scope of this review, which focuses on the most accessible and reproducible markers used in daily clinical practice. Nevertheless a brief mention is made for potential subsequent studies into their prognostic value in patients with HCC undergoing LT.

However, one particular biomarker worthy of mention is glypican-3 (GPC3). This is a membrane glycoprotein which is involved in cell cycle regulation and which is detected in HCC patients. Although there is as yet no unanimous agreement on this, high levels of GPC3 in HCC tissue after curative resection and LT seem to lead to poor prognosis in terms of both disease free and overall survival[98,99]. In addition, a link has been found between high GPC3 expression and high tumor grade (moderate and poor differentiation), late TNM stage (Ⅲ, Ⅳ), vascular invasion, tumor multifocality and metastasis in patients with HCC. Research has also shown high GPC3 expression to be associated with the presence of large tumors (5 cm or more)[100]. The importance of glypican 3 in patients with HCC undergoing liver transplantation has already been proved to be useful in prognosis stratification and several authors propose a cut-off value of 3.5×10^{-2}[101,102].

Another important biomarker to mention is human telomerase reverse transcriptase mRNA (h-TERT mRNA). Several studies have demonstrated that high h-TERT mRNA expression is a prognostic indicator of poor outcome in HCC patients. The prognostic power of h-TERT mRNA has been evaluated also in a liver transplant setting: HCC patients with an elevation of human telomerase reverse transcriptase mRNA (preoperatively in the blood or after neoadjuvant immunochemotherapy) suffered of higher tumor recurrence and lower survival rates than those without h-TERT mRNA in the blood[103,104]. More studies are required to validate the prognostic power of h-TERT mRNA due to an absence of an unanimous consensus[105,106].

High levels of alpha 1 fucosidase (AFU) and transforming growth factor beta-1 (TGF-B1) seem to be associated with poor prognosis in patients with HCC. Although more studies are required to evaluate the importance of said biomarkers after LT[107-113].

Furthermore, biomarkers such as human cervical cancer oncogene (HCCR), tumor specific growth factor (TSGF) and gamma-glutamyl transferase Ⅱ (GGT Ⅱ) have been proved to have an important role in diagnosis, but more studies are required to clarify its prognostic role[56,114-120].

Systemic inflammatory markers

Systemic host inflammation is another factor that has been evaluated as a parameter to assess tumor aggressiveness[121,122]. Depending on the tumor micro-environment, pro-inflammatory macrophages, cytokines and chemokines seem to be factors responsible for tumor progression given their ability to induce metastatization and to inhibit apoptosis, thus facilitating angiogenesis and DNA damage[123].

Neutrophil-to-lymphocyte ratio (NLR) is a serum inflammatory marker that has been attracting increasing interest since it has been found to be a predictor of recurrence and poor prognosis in patients with colorectal-liver metastasis[123-125]. High neutrophil levels are able to enhance the propensity for vascular invasion and metastatization by increasing the production of VEGF[126,127]. Conversely, low lymphocyte numbers seem to be responsible for impaired immunosurveillance against disease development and progression[128].

In 2009, Halazun *et al*[123] demonstrated that NLR predicts outcome in HCC patients after LT. They reported that patients meeting the Milan criteria with a NLR $\geqslant$ 5 had significantly worse recurrence-free survival (RFS) and lower survival rates than patients with a NLR < 5. Similar effects on tumor recurrence and survival were later reported by other groups both for cadaveric and for living donor liver transplantation (LDLT)[129-132]. A recent meta-analysis evaluating the prognostic significance of NLR in HCC patients confirmed, moreover, that high NLR was associated with poor OS and DFS of liver transplanted patients with HRs of 3.42 (2.41-4.85) and 5.90 (3.99-8.70), respectively. Notably, conventional prognostic indexes such as vascular invasion, multiple tumors, and AFP

⩾ 400 ng/mL were also reported to be correlated with NLR[133]. Despite discordant findings[69,134], the data uncovered until now suggest that NLR can contribute to the LT selection process of HCC patients. Further data are needed to confirm the marker's effectiveness.

Other inflammatory-related prognostic markers are under evaluation. In a retrospective intention-to-treat analysis on 181 HCC patients listed for LT, Lai *et al*[135] demonstrated that the platelet-to-lymphocytes ratio (PLR) > 150 was more efficacious than NLR in predicting the risk of HCC recurrence after LT and that it can be used to stratify patients for tumor-free-survival (91.6% *vs* 80.7%, *P* = 0.02). The usefulness of PLR was recently confirmed by a Chinese study focusing on a cohort of 343 HCC in whom a PLR = 125 was found to be the most appropriate cut-off to predict tumor-free survival after LT (sensitivity 61.6%, specificity 62.7%)[136]. Unanimous agreement has, however, yet to be reached[134].

Some have hypothesized that an inflammatory response is implicated in the pathogenesis of cancer-related malnutrition[137]. The prognostic nutritional index (PNI) has been proposed as a further marker of inflammation and HCC-related prognosis. Chan *et al*[138] demonstrated that PNI is an independent prognostic index of OS and DFS after surgical resection of Barcelona Clinic Liver Cancer (BCLC) Stage 0/A, but its potential role in the liver transplant setting is as yet unestablished.

Due to a clear lack of single, self-sufficient prognostic biomarkers, some attempts have been made to assess prognosis using an integrated combination of more than one of these. Toyoda *et al*[139] for example, used an Asian population to develop the so-called BALAD staging score which is based on 5 serum markers including AFP, AFP-L3% and DCP; the scoring system's predictive power was found to be similar to that of the BCLC staging system . Although the model was recently validated on a British population[140], it has yet to be validated in a transplant setting.

Retrospectively studying 185 patients who underwent hepatectomy for HCC, Kiriyama concluded that triple positive tumor markers for HCC (AFP, AFP-L3% and DCP) correlated with the poorest prognosis and the most invasive characteristics in pathological findings[141].

As a final consideration, research on miRNA plasma expression is arousing interest in view of the potential role of miRNA signature profiling in HCC prognosis stratification[142].

RADIOLOGICAL FEATURES: THE ROLE OF ^{18}F-FDG-PET IMAGING

Morphological imaging studies have proven to be effective in predicting outcome after orthotopic liver transplantation (OLT). While both the Milan and the UCFS criteria are based on the size and number of radiologically detected tumors, functional imaging studies appear to be able to provide even further information about the tumor. F-18 fluoro-2-deoxy-d-glucose positron emission topography (^{18}F-FDG PET) estimates the tumor growth and metabolism based on calculated tumor volumes and maximum standardized uptake values. The different ratio of glucose-6-phosphatase and hexokinase in the normal liver and tumor cells results in an increased accumulation of ^{18}F-FDG in primary HCC lesions[143].

While ^{18}F-FDG-PET has demonstrated suboptimal sensitivity in detecting new HCC (< 50%)[144], there are reports that it is useful in uncovering the presence of extra-hepatic metastasis[145], in providing information about HCC prognosis, and in predicting tumor recurrence after LT[146-149]. In a retrospective analysis, Yang *et al*[146] demonstrated that ^{18}F-FDG-PET positive patients (PET +) showed an overall greater risk of tumor recurrence with respect to PET negative (PET-) patients (OR = 7.6). Its ability to predict prognosis and tumor recurrence was confirmed in 2009 by Kornberg *et al*[147] who carried out a retrospective analysis on 42 liver transplanted patients. Their results demonstrated that PET+ patients had a significantly worse 3-year DFS (35%) and a higher recurrence rate (RR 50%) than PET- patients (DFS = 93%, RR = 3.8%). The same research group recently demonstrated that HCC patients meeting the Milan criteria with a non-avid ^{18}F-FDG PET achieved an excellent DFS after LT (> 80% at 5-year follow-up). An avid uptake of ^{18}F-FDG was found to be an independent predictor of tumor related drop-out from waiting lists. This finding confirms the potential advantages of this technique in the LT setting[148].

As far as biological findings are concerned, Yang *et al*[146] demonstrated that PET^{+} (greater PET lesion uptake) HCCs were significantly associated with some poor prognostic factors such as AFP > 200 ng/mL (*P* < 0.001) and vascular invasion (*P* = 0.003)[146]. Kornberg likewise demonstrated that PET+ status was an independent predictor of microvascular invasion. The uptake of ^{18}F-FDG in HCC patients is reported, moreover, to vary according to the degree of tumor differentiation[146]. Well- and well-to-moderately differentiated HCCs, in particular, exhibit an ^{18}F-FDG metabolism that is similar to normal liver tissue while moderate-to-poorly and poorly differentiated HCCs demonstrate an enhanced one[146,150,151].

New tracers (*e.g.,* ^{18}F-fluoropropyloxy-L-tryptophan and L-methyl-^{3}H-methionine, ^{11}C-metomidate, ^{11}C-acetate, ^{18}F-fluorocholine and ^{11}C-choline) aiming to improve PET's specificity and sensitivity in detecting HCC and its metastases are presently under investigation[152-154]. Dual Tracer PET-CT imaging is also under examination: ^{11}C-acetate and ^{18}F-FDG have already demonstrated high sensitivity and specificity in detecting HCC (about 95% and 100% respectively) in candidates for LT or liver resection[155,156]; the potential role of these new tracers and the recently introduced oncologic PET-MRI[157] in the

transplant scenario and their ability to detect biological aggressiveness remain to be established.

HISTOLOGY: THE PRE-TRANSPLANT LIVER BIOPSY

As mentioned above, microvascular invasion is an established independent prognostic factor for HCC recurrence after LT[2-7]. A strong correlation between microvascular invasion and the histologic tumor grade of HCC has, in fact, been reported by many authors[2,14,19,158,159] , and high tumor grade has been found to be an independent predictor of vascular invasion[160]. In addition, Tamura *et al*[42] reported that histologic differentiation itself represents an independent predictor of survival following transplantation since a low tumor grade increases the 3-year survival rate both in patients with small (≤ 5 cm) and large (> 5 cm) tumors.

A very low post-transplant tumor recurrence rate was found by our group when a pre-transplant biopsy grade-based selection protocol that did not include the patient's Milan criteria standing was utilized in 145 HCC patients. When the G3 HCCs were excluded from LT, the 5 year survival free recurrence was 92% and none of the patients with tumors > 5 cm had recurrence[19]. When poorly differentiated HCCs were excluded from our patient database, only 12% of the tumors were > 5 cm which, of course, confirms the relevance of a tumor grading selection system. DuBay *et al*[161] who subsequently validated this approach, studied 294 HCC patients for 8 years during which time they gradually tested and developed a transplant selection protocol. Their findings showed results gradually shifting away from the Milan criteria and towards a biopsy-based system (the extended Toronto criteria). A comparison between the two periods of the study confirmed that the exclusion of poorly differentiated tumors irrespective of macromorphological features (Milan criteria) achieved excellent survival rates.

Findings from Pawlik *et al*[162] single-center study did not, however, confirm the independent prognostic power of a pre-operative biopsy for outcome after LT. While the tumor grade associated with vascular invasion or disease-specific death was not significant, the tumor grade on the final pathologic examination was found to be a reliable predictor of vascular invasion and outcome. The disparity between the preoperative histology and the final surgical specimen analysis may be explained by the fact that well or moderately-differentiated tumor areas can coexist adjacent to poorly differentiated ones. Sampling bias should, therefore, always be taken into consideration. The median tumor size in Pawlik *et al*[162] study was 7.0 cm, and half of the patients had very large tumors with a markedly increased risk of heterogeneity and dedifferentiation[163,164]. More generally, while the biopsy reading is strictly related to the quality of the tissue sample, the histologic grade is subject to inter-observer variability. Notwithstanding the existence of the Edmondson and Steiner grading system[165], and although automated grading systems have been proposed[166], HCC biopsy imaging grading is still visual, qualitative, and subjective. Indeed, the inter-observer variability has been shown to be relevant, with a K statistic from 0.32 to 0.66, respectively, for moderately and well differentiated HCC[167]. Lack of concordance clearly limits the widespread use of a grade-based selection protocol of HCC patients for LT.

On the other hand, if one goes beyond the possibility of "false negative" results, virtually all G3 biopsies in HCC patients refer to true poorly differentiated tumors, and all high grade tumors prove to be strictly correlated with poor outcome after LT. Excluding these patients from LT listing may contribute to reducing the prevalence of patients with aggressive HCC and this, of course, will lead to beneficial results in terms of overall post-transplant outcome, as has been reported by one of our studies as well as by a Canadian study.

Multiple fine needle aspiration biopsies (FNABs) are not, however, recommended given the complication rates that vary from 0.75% to 13.6%[168]. Cases of bleeding have also been described in 1/500 biopsies and those requiring urgent hospitalization and blood transfusion range from 1 /2500 to up to 1/10000 biopsies. There is, in addition, a 0 to 3% risk of needle tract seeding in most studies although it did not seem to influence the oncological outcome[169-171].

A visual liver and biopsy site assessment could overcome these sampling issues. Core needle biopsy (CNB) under laparoscopic ultrasonography (LUS) guidance makes it possible to directly examine the area to be sampled[172,173]. Helmreich-Becker *et al*[174] described LUS guided CNB as a safe and feasible procedure. LUS appears to be extremely promising tool in the transplant setting; biopsy specimens and high diagnostic accuracy can be obtained, and HCC can be treated (downstaging - bridging) all at the same time and potentially repeatedly. Further studies are needed to establish its role in the pre-transplant assessment of HCC patients.

While current guidelines do not include liver biopsy to diagnose radiologically typical[175] HCC, given the tendency to characterize tumors focusing on their molecular features, biopsies will presumably play a key role in the near future. Large steps forward have, in fact, been made in the molecular signatures field thanks to the development of microarray technologies which permit the tumor expression of several molecular markers associated with deregulation of genes or pathways to be tested at the same time by processing a tumor tissue sample[176,177]. Deregulation of these genes and pathways has, in turn, been proven to affect some of the tumor's biological features such as vascular invasion[178,179] and growth rate[180,181],

and several studies have shown that it plays a role in worsening prognosis[181-184]. In a recent paper, Villa *et al*[181] demonstrated that a five-gene trascriptomic hepatic signature including angiopoietin-2, NETO2, DLL4 ESM1 and NR4A1 was able to rapidly identify growing HCCs and was independently associated with an increase in mortality. Several signatures, including the miRNA expression pattern[185] have been proposed and studied until now, but agreement on the best predictive molecular signature pattern has yet to be reached.

It is worth mentioning that epigenetic features have also been investigated as prognostic biomarkers. In fact, as with other cancers, HCC has demonstrated a distinct methylation profile. The hypermethylated form of p16 (CDKN2A), for example, a tumor-suppressor gene involved in cell cycle regulation, has already been associated with advanced stages of HCC, vascular invasion, poor tumor differentiation and, finally, with worse prognosis[186].

In view of its relatively recent appearance, the molecular signature still lacks clinical relevance. Evidence gathered until now suggests that it can be potentially used in the LT evaluation workup. Even more importantly, in the same way that a great deal of information can be gained from a biopsy specimen, the same can be said for the tumor's histological features. In addition, recent technologies have made it possible to obtain genomic profiling on formalin fixed, paraffin embedded samples making the molecular signature a feasible, reproducible tool for tumor evaluation in the near future[184].

RESPONSE TO THERAPY

Loco-regional therapy response in LT candidates with HCC has been extensively studied over the last 10 to 15 years, but an in-depth analysis of the topic does not fall within the aims of the current work[15,29,175,187-200]. Despite the fact that there is a paucity of randomized clinical trials regarding downstaging or bridging therapies, most centers throughout the world adopt loco-regional therapies before or after placing a patient on a waiting list[188,196,201-203].

Resection, ablation (either percutaneous or laparoscopic), or TACE alone or together with other therapies, which are the most widely used therapeutic strategies, aim to reduce the dropout rate while patients are on waiting lists and/or tumor recurrence after LT[204-206]. Although downstaging strategies focus on reducing the tumor burden until the patient meets transplantation criteria (*i.e.,* Milan), it is widely accepted that a prolonged response to downstaging therapies can itself be considered a selection criteria that mirrors the biologic behavior of the tumor and predicts a relatively low risk of recurrence after LT. Similarly, as reported by a number of studies, a good response to bridging therapies can serve as a surrogate marker of a favorable tumor biology (Table 3).

In an intention-to-treat analysis, Millonig *et al*[187] demonstrated that patients with a complete response to TACE had 1-, 2-, and 5-year survival rates of 89.1%, 85.1%, and 85.1%, respectively, compared with 68.6%, 51.4%, and 51.4% in non-responders (P = 0.02). Similar results were also found by our group while studying recurrence after LT in patients who were stratified into responder and non-responders to pre-transplant HCC therapy. The probability of post-transplant HCC recurrence was shown to be higher in the non- responder (P = 0.04) group[15]. In a seminal study by Otto *et al*[207] 136 HCC patients who underwent TACE were assessed during the waiting period for LT in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. The authors reported a 22% 5-year freedom from recurrence rate in the TACE-with-progression group and a 92% rate in the TACE-without-progression one (P < 0.0001). Response to therapy was also more predictive of outcome than was the Milan criteria (P = 0.0001).

Different 5-year recurrence rates (19.4% in patients with partial or no response to bridging therapy *vs* 5.5% in responders) were also detected by Cucchetti *et al*[199] who studied the data of 315 LT candidates. A strict correlation between tumor responsiveness and outcome was also found when progression or no-progression was found at the pathological examination[208,209]. Ho *et al*[210] assessed loco-regional treatment (both as downstaging or bridging strategies) before LT in 86 HCC patients by dividing the population into 3 subgroups depending on the degree of tumor necrosis at the pathological examination (group Ⅰ: 10%-50%; group Ⅱ: 50%-90%; and group Ⅲ: > 90). The patients with a higher necrosis rate after therapy (group Ⅲ) were found to survive longer (P = 0.003) and had significantly lower recurrence rates than the patients in the other two groups (P = 0.001). Finally, when response to therapy was integrated with higher AFP levels, its power to predict prognosis in terms of RFS and OS was found to be enhanced. Absence of progression as far as tumor burden and the AFP (slope ≤ 15) were concerned identified a subgroup of patients with excellent prognosis, irrespective of conventional criteria[211] (Table 2).

With regard to downstaging, some studies have shown that the outcome of downstaged HCCs patients was similar to that of patients meeting the Milan criteria. Ravaioli *et al*[17] reported a 3-year RFS in patients fulfilling the Milan criteria and in downstaged patients of 83% and 75%, respectively (P = NS). As outlined in Table 3, similar results were found by Chapman *et al*[212] and De Luna *et al*[213]. Yao *et al*[214] recently reported long term results after LT in HCC patients downstaged to T2 and compared their data to those of patients meeting the Milan criteria: the 5-year post-transplant and recurrence-free survival were 77.8% and 90.8%, respectively, in the downstaged group *vs* 81% and 88% in the T2 group (P = NS). Since the

Table 3 Response to therapy: comparison of outcomes following different pre-transplant strategies

Ref.	Treatment	Response assessemnt	Transplant criteria	No. of patients		Outcome		Comparison between responders and non responders	Comparison between downstaged patient *vs* conventional criteria
Millonig *et al*[187]	TACE	RECIST	UCSF	Total	116	Total	-		
				Downstaging (DS)	NA	DS responders	5-yr OS = 25%		NS[1]
				Bridging (B)	NA	B responders	5-yr OS = 85.7%	0.02	
						B non responders	5-yr OS = 51.4%		
Chapman *et al*[212]	Resection, ablation, TACE	RECIST	Milan	Total	136	Total	-		
				DS	76	DS responders	5-yr RFS = 50%		NS
				B	60	B responders	5-y RFS = 62.6%	NA	
						B non responders			
Vitale *et al*[15]	Resection, ablation, TACE	RECIST	Milan	Total	147		5-yr ITT survival = 74%		
				DS	NA	DS responders	-		NA
				B	NA	B responders	5-yr ITT survival = 83%	< 0.01	
						B non responders	5-yr ITT survival = 63%		
Cucchetti *et al*[199]	Resection, ablation, TACE	mRECIST	Milan	Total	315	Total	-		
				DS	53	DS responders	5-yr RR = 19.2%		NS[1]
				B	240	B responders	5-yr RR = 5.5%	0.017	
						B non responders	5-yr RR = 19.4%		
Ravaioli *et al*[17]	Resection, ablation, TACE	RECIST	Milan	Total	177	Total	3-yr RFS = 82%		
				DS	48	DS responders	3-yr RFS = 75%		NS
				B	NA	B responders	3-yr RFS = 83%	NA	
						B non responders			
Yao *et al*[214]	Resection, ablation, TACE	mRECIST	Milan	Total	606	Total	-		
				DS	118	DS responders	5-yr RFS = 90.8%		NS
				B	NA	B responders	5-yr RFS = 88%	NA	
						B non responders			
De Luna *et al*[213]	TACI	NA	Milan	Total	122	Total	3-yr OS = 82.7%		
				DS	27	DS responders	3-yr OS = 84.1%		NS
				B	NA	B responders	3-yr OS = 84.7%	NA	
						B non responders			
Graziadei *et al*[190]	TACE	NA	Milan	Total	63	Total	NA		
				DS	15	DS responders	4-yr OS = 41%		NA
				B	48	B responders	5-yr OS = 94%	NA	
						B non responders	na		
Otto *et al*[195]	TACE	mRECIST	Milan	Total	136	Total	-		
				DS	49	DS responders	5-yr RFS = 92%	< 0.0001	NA
				B	87	B responders	5-yr RFS = 22%		
						B non responders			

De Giorgio *et al*[198]	Resection, ablation, TACE	NA	Milan	Total DS B	206 NA 83	Total DS responders B responders B non responders	NA	NA	NA

[1]Computed not stastically significant. NA: Not available; NS: Not statistically significant; OS: Overall Survival; RFS: Recurrence free survival or freedom from recurrence; RR: Recurrence rate; RECIST: Response Evaluation criteria in solid tumors; mRECIST: Modified response evaluation criteria in solid tumors; TACE: Trans arterial chemo-embolization; TACI: Trans Arterial chemo-infusion; UCSF: University of California San Francisco.

2-year cumulative probability for dropout was 34.2% in the downstaged group *vs* 25.6% in the T2 LT recipient group, this suggests that downstaging has an important impact on tumor biology. Interestingly, the prevalence of microvascular invasion and of poorly differentiated grade was found to be similar in the two groups when the final pathological features were compared.

These findings provide further evidence that non-responsive HCCs could conceal an aggressive biology while responsive ones could mirror a milder behavior. At the same time, they emphasize the relevance of response to therapy as an index of tumor biologic aggressiveness and the need to develop standardized guidelines to evaluate it. Several studies suggest using the modified version of Response Evaluation Criteria In Solid Tumours (mRECIST)[215-218] which constitute the modified version of the RECIST criteria for the assessment of response to therapy in solid tumors[219]. With respect to conventional criteria, modified ones include an oncological assessment of tumor viability and focus on reducing the viable tumor volume, defined by enhanced areas on imaging. Standardized criteria could help to improve homogeneity in response to therapy assessments.

There are, nevertheless, still some limitations with regard to the reliability and reproducibility of mRECIST. Since tumor assessment is subjective, its accuracy depends both on the technician's ability as well as on the quality of imaging[220]. Furthermore, its criteria are only applicable to HCC with typical features because assessment of response in atypical HCC remains obscure[220]. Finally, vascular shunt and alterations (especially for infiltrative tumors) can alter enhancement and thus lead to interpretation errors. In view of these and other considerations, some authors suggest evaluating AFP variations together with radiological features for an objective assessment of response to therapy[47,211].

A potential drawback of bridging and downstaging therapies is, however, tumor dedifferentiation. Kojiro *et al*[221] reported that tumors with sarcomatoid changes were more frequent in patients who were treated with TACE before transplant than those who were not. Sarcomatoid modification has also been described in some case reports after radiofrequency ablation[222,223]. Zen *et al*[224] reported that only pretreated HCCs showed dedifferentiation towards a biliary phenotype when they analyzed explanted specimens. Yamamoto *et al*[225] reported a high recurrence rate after radiofrequency ablation (RFA) in tumors that had aggressive biological features.

Selection pressure on resistant cells, phenotypic adaptative changes, and the protein expression normally trigged by hypoxia may explain therapy-induced histopathological changes[224,226]. Regardless of etiology, caution should be used when applying pre-LT therapies as investigation tools given the biological switch phenomenon.

Test of time

A complete or partial response to therapy does not guarantee that it will be stable over time, and a rapid recurrence after response could uncover an aggressive tumor biology which would contraindicate the transplant. Not only the assessment of the response to therapy but also the course following that response (or more generally the overall tumor growth rate) are important considerations when HCC patients are being evaluated for LT as far as the so-called "test of time" is concerned. Reporting good outcomes when tumors were closesly evaluated for 8 mo from the time of ablation to the date of enlisting a patient for transplant[210] , Roberts *et al*[210] proposed scheduling a waiting period following downstaging procedures to assess tumor behavior. Toso *et al*[227] also stressed the importance of an observation period between entering a downstaging program and being placed on a transplantation waiting list and suggested utilizing at least a 6-mo minimum test of time. Cescon *et al*[122] instead, proposed using a 3-mo waiting period with re-staging at the end to verify the new status. Despite the drawback of having to wait and the intrinsic increase in drop-out risk, the test of time appears to be an efficacious surrogate marker of tumor aggressiveness that could be integrated with other "static" prognostic tools (histology, response to therapy, morphologic studies). Further studies using standardized response assessments and homogeneous periods are needed to evaluate the parameter's true potential.

VOLUME DOUBLING TIME

In 1961 Mordecai Schwartz proposed a biomathematical approach to clinical tumor growth and the formula he outlined to calculate the oubling time (DT) was:

$$DT = t \ln 2/(\ln V2 - \ln V1)$$

Where t is the time interval between measurements and V2 and V1 are the tumor volumes detected at imaging, respectively, at the end and at beginning of the time interval[228].

Subsequent studies described a wide variability in DTs with values ranging from < 30 d up to 600 d[229-234]. The assumption that various growth velocities reflect different tumor behaviors led researchers to search for correlations between HCC DT and other tumor or patient characteristics. The close relationship between DTs and prognosis has recently been investigated by Villa *et al*[181] in a prospective study on 78 patients with newly diagnosed HCC. Study data confirmed that tumor doubling time ranged from 30 to 621 d. When the study population was divided into quartiles according to HCC growth rate, different survival profiles depending on the speed of tumor DT were found: 25% of the patients demonstrated DTs less than or equal to 53 d and had a significantly worse prognosis than the patients with DTs in other quartiles, regardless of the treatment prescribed[181].

Static macromorphological parameters such as initial tumor diameter and ultrasound features have been demonstrated to be correlated with DT[231,234]. More interestingly, however, DT seems to be correlated with tumor differentiation[229,231,232], mitotic activity[229,230], vascular invasion and direct indexes of biological aggressiveness. Nakajima *et al*[229] studied 34 patients with small HCCs using some markers of cell division such as Ki-67, Apo-I and their histologic grade to classify the tumors as slowly, moderately, or rapidly growing. They concluded that the more rapid the tumor growth, the higher the cell production and the less differentiated the tumor[229]. Moreover, as reported by several studies[232,234-236], AFP levels were found to be correlated with tumor growth velocity, and this finding has confirmed the indirect link between DT and biological aggressiveness.

In addition, the direct influence of DT on outcome after surgery has also been reported. Okazaki *et al*[230] outlined poor outcomes after hepatectomy in those patients whose DT was short. Similarly, Cucchetti *et al*[232] calculated that the 3-year recurrence rate after liver resection was significantly higher in patients with DT < 100 d than in those with DT > 100 d ($P = 0.008$). Even if there are no reports on the effect of DT on the post-transplant outcome, the studies mentioned above clearly demonstrate that a tumor's growth velocity is a faithful mirror of its intrinsic aggressiveness. It seems reasonable then, although there are no studies to prove it, that rapidly growing tumors have poor outcomes after LT.

While the strict correlation between AFP levels and tumor growth velocity has been repeatedly demonstrated[232,234-236], the lack of agreement about this link[237] and the ease of obtaining the DT parameter point the way to further research on the role of DT in the evaluation of HCC aggressiveness in LT candidates.

CONCLUSION

Predicting post-transplant HCC recurrence on the basis of the tumor size and the number of nodules can only seem simplistic and imprecise in the light of the disease's complexity. A number of recent studies have confirmed the predictive accuracy of other parameters used to assess the biological behavior of HCC and in particular with reference to tumor progression and response to therapy. Prospective randomized studies designed to validate the prognostic role of each of these parameters present relevant feasibility issues. Repeatable, multiparametric, integrated models develped on the basis of large multicentric prognostic studies are no doubt the best strategy to improve our ability to select the most appropriate HCC patients for liver transplant.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Current status and perspectives of immune-based therapies for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a frequent cancer with a high mortality. For early stage cancer there are potentially curative treatments including local ablation, resection and liver transplantation. However, for more advanced stage disease, there is no optimal treatment available. Even in the case of a "curative" treatment, recurrence or development of a new cancer in the precancerous liver is common. Thus, there is an urgent need for novel and effective (adjuvant) therapies to treat HCC and to prevent recurrence after local treatment in patients with HCC. The unique immune response in the liver favors tolerance, which remains a genuine challenge for conventional immunotherapy in patients with HCC. However, even in this "immunotolerant" organ, spontaneous immune responses against tumor antigens have been detected, although they are insufficient to achieve significant tumor death. Local ablation therapy leads to immunogenic tumor cell death by inducing the release of massive amounts of antigens, which enhances spontaneous immune response. New immune therapies such as dendritic cell vaccination and immune checkpoint inhibition are under investigation. Immunotherapy for cancer has made huge progress in the last few years and clinical trials examining the use of immunotherapy to treat hepatocellular carcinoma have shown some success. In this review, we discuss the current status of and offer some perspectives on immunotherapy for hepatocellular carcinoma, which could change disease progression in the near future.

Key words: Hepatocellular carcinoma; Immunotherapy; Dendritic cells; Dendritic cell vaccination; Therapy

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Core tip: Hepatocellular carcinoma is a frequent cancer with a high mortality. For early stage cancer there are potentially curative treatments including local ablation, resection and liver transplantation. However, recurrence or development of a new tumor after treatment are not uncommon. Moreover, for more advanced stage disease, there is no optimal treatment available. Thus, there is an urgent need for novel and effective therapies for advanced stage hepatocellular carcinoma, and to prevent and to treat recurrence after local treatment of hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and fatal cancers in the world. Men are more often affected than women, with 554000 and 228000 new cases per year, respectively. It is the second most common cause of death from cancer worldwide, leading to 746000 deaths in 2012. Viral hepatitis B and C, chronic alcohol consumption and non-alcoholic fatty liver disease are major risk factors[1].

Once diagnosed, HCC frequently has a dismal prognosis because of the low effectiveness of available treatments. The choice of treatment is selected according to the Barcelona Clinic Liver Cancer staging system, which integrates tumor characteristics and performance status with liver function and links them to evidence-based therapeutic option[2]. Early and very early stage tumors are potentially curable with surgery (resection or liver transplantation) or local therapies including radiofrequency ablation (RFA) or percutaneous ethanol injection. The 5 year survival rate in these patients ranges from 40% to 70%[2]. In the absence of liver transplantation, tumor recurrence is observed in 70% of patients after resection or RFA after a time period of 3 years. Unfortunately, less than 30% of HCC patients are eligible for these procedures because most have intermediate or advanced stage disease at diagnosis (large or multifocal tumors, or liver insufficiency, which limits treatment). For intermediate stage tumors, transarterial chemoembolization (TACE) (conventional or drug eluting beads) has become the standard of care[3]. However, there is certainly room for improvement because the 3-year survival rate is only approximately 60%[4,5]. In advanced stage disease, the oral multi-targeted kinase inhibitor sorafenib offers a survival benefit of approximately 3 mo[6]. Other molecules, such as sunitinib or linifanib have not been proven to be superior to sorafenib[7,8]. Several newer molecules have been shown to confer a survival advantage in a subset of patients[9]. The mean survival of patients with advanced stage HCC is less than 1 year.

Recurrence rates remain high in very early to intermediate stages despite the availability of potentially curative treatment. There are 2 main reasons responsible for this phenomenon. First, a small tumor that is undetectable using current imaging modalities may exist before treatment and would thus be left untreated; second, a new tumor may occur in the diseased liver, which can be considered a pre-neoplastic organ. The efficacy of combining local treatment with systemic treatment has been studied in several clinical trials. In phase Ⅰ and Ⅱ trials, the preliminary results from using combination therapies were promising. However, two large multicenter phase Ⅲ trials studying the effect of sorafenib after TACE (SPACE study)[10] or surgical resection/RFA (STORM study)[11] failed to demonstrate any adjuvant effect of sorafenib on survival. This underlines the need for novel and effective adjuvant therapies to treat patients with HCC and to prevent recurrence after local treatment.

IMMUNE RESPONSE IN THE LIVER AND IMMUNOTHERAPY

The liver is a unique organ in several ways, one of which is its blood supply. Approximately 25% and 75% of the blood enters the liver through the hepatic artery and portal vein, respectively[12]. After a meal, the percentage of the portal vein supply increases further. The portal vein drains into smaller diameter structures, known as the sinusoids. Vascular resistance is very low in these structures, and portal venous blood, which is loaded with food and microbial antigens from the intestine, flows extremely slowly in the sinusoids. Moreover, liver sinusoidal cells (LSECs) are fenestrated thus facilitating the passage of cells and antigens between the sinusoids and the space of Disse, which is in contact with hepatocytes. With the exception of hepatocytes and biliary cells, the liver hosts a number of non-parenchymal cells including LSECs, hepatic stellate cells, Kupffer cells, dendritic cells (DCs), and lymphocytes. All of these cells play roles in the barrier function of the liver, separating it from the gastrointestinal tract and the rest of the body. Indeed, DCs, LSECs, hepatic stellate cells and Kupffer cells are all able to present antigens to antigen-specific lymphocytes. These cells are resident cells and induce tolerance rather than immunity. For a more detailed discussion of the immunological properties of the liver, we refer the reader to several recent, excellent reviews on this topic[13-15].

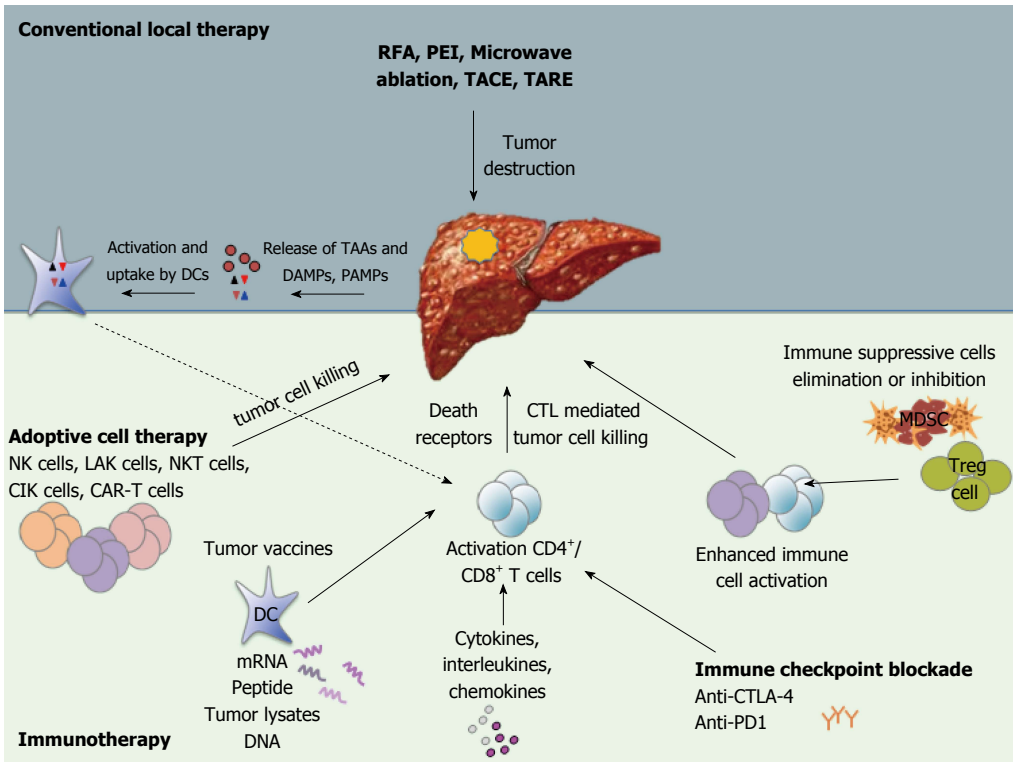

Figure 1 Immune-based therapies for hepatocellular carcinoma. Tumor-specific T cells can be stimulated by several ways including adoptive cell therapy, tumor vaccines, cytokines or by inhibiting immune suppressive mechanisms (immune checkpoint inhibitors, depletion of Tregs or MDSCs). These tumor-specific T cells are able to kill the tumor cells in an antigen-specific way. Conventional therapies (RFA, TACE, PEI, microwave ablation, TARE) can destroy tumor cells, which result in the release of tumor antigens and danger-associated molecular patterns that can activate DCs resulting in the activation of tumor-specific T cells. RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization; DAMPs: Danger-associated molecular patterns; PAMPs: Pathogen-associated molecular patterns; DC: Dendritic cell; NK: Natural killer; LAK: Lymphokine-activated killer; NKT cell: Natural killer T cell; CIK: Cytokine-induced killer; CAR: Chimeric antigen receptor; CTL: Cytotoxic T lymphocyte; Treg: Regulatory T cell; MDSC: Myeloid derived suppressor cells; CTLA-4: Cytotoxic T lymphocyte antigen 4; PD-1: Programmed-death 1.

Immunotherapy for cancer is based on harnessing the potential of the immune system to destroy malignant cells. However, immune responses in the liver might be problematic, as the liver mainly induces tolerance. The liver is a distinctive organ with respect to immune function and possesses a unique form of immune regulation: immune tolerance is induced to avoid chronic inflammation caused by antigens present in portal vein blood. This might prevent an adequate immune response against malignant cells. Indeed, it has been shown that an increased quantity of circulating regulatory T (Treg) cells in patients with HCC is associated with a high mortality rate and reduced survival[16]. Overcoming this immune tolerance is thus an important challenge in the search for an effective immunotherapy against HCC. On the other hand, there have been reports of spontaneous regression of HCC associated with tumor hypoxia or systemic inflammatory response[17]. Additionally, the regression of HCC has been described following the discontinuation of immunosuppressive therapy[18]. Therefore, immunotherapy remains an attractive approach for boosting the immune system in patients with HCC to enhance the efficacy of current therapies (Figure 1).

SPONTANEOUS TUMOR-SPECIFIC IMMUNE RESPONSE IN THE LIVER

Immune evasion is a characteristic of cancer and is even more prevalent in organs with high immune tolerance such as the liver. High levels of $CD4^+CD25^+FOXP3^+$ Treg cells in peripheral blood are an independent predictive factor of poor survival after TACE treatment in patients with HCC[19]. In addition, the presence of high numbers of intrahepatic $CD8^+FoxP3^+$ regulatory T cells was found to be associated with more advanced stages of HCC[20]. Additionally, there is an increased presence of myeloid-derived suppressor cells is increased in patients with HCC compared to healthy donors[21].

Even if HCC is not generally considered an "immunogenic" tumor type, immune responses do occur in livers that have been invaded by HCC. In a number of patients, spontaneous immune responses against tumor antigens have been detected, but these were insufficient to achieve a significant therapeutic effect[22-24]. Moreover, naturally occurring tumor-associated antigen-specific T-cell responses exist in patients with HCC and correlate with patient survival[25]. Patients whose tumors express multiple tumor-associated antigens (TAAs) and contain TAA-specific

$CD8^+$ T-cell lymphocytic infiltrates show longer survival rates and a lower risk of recurrence[25-27]. A strong $CD8^+$ T-cell response against TAAs was shown to improve recurrence-free survival after surgery.

Over the past 15 years, a number of TAAs have been identified in HCC, some of which elicit tumor-specific immune responses[24]. Most of the identified TAAs are not specific to HCC, but some HHC-specific TAAs are targeted by T cells, which makes them good potential targets for immunotherapy; these include alpha-fetoprotein (AFP), glypican 3 (GPC3), melanoma antigen gene A (MAGE-A), and New York-esophageal squamous cell carcinoma-1 (NY-ESO-1)[25,28].

AFP, which is expressed in the fetus and repressed after birth, is re-expressed in the majority of HCCs. Even if it has a low sensitivity and specificity as a serum marker, it continues to represent a useful clinical marker for HCC[29]. In some studies, HCC patients had increased frequencies of circulating AFP-specific $CD8^+$ T cells[23,30].

GPC3 is a cell surface heparin sulfate proteoglycan. GPC3 mRNA expression is low or absent in normal liver tissue, in benign tumor lesions (such as focal nodular hyperplasia) and in a cirrhotic liver[31]. However, GPC-3 is detected in approximately 80% of HCCs even in the early stages[27,32]. GPC3-specific cytotoxic T-lymphocytes have a high level of killing activity against HCC tumor cells. A study from Nobuoka *et al*[33] showed that GPC3 has strong immunogenicity.

MAGE-A was first described in melanoma, but it has been shown to be widely expressed in various tumors. In cirrhotic patients with HCC, mRNA encoding MAGE-1 was found to be present in 80% of resected HCCs[34]. MAGE-A and SSX-2 specific $CD8^+$ T cells were found to be enriched in HCC, but not in surrounding liver tissue[35]. In healthy subjects, NY-ESO-1 is only expressed in testes, but it is expressed in several tumors, including HCC. NY-ESO-1-specific $CD8^+$ T cells have also been shown to be present in HCC[36,37].

Flecken *et al*[25] studied the frequency and tumor-infiltration capacity of naturally occurring $CD8^+$ T-cell responses targeting AFP, GPC3, MAGE-A and NY-ESO-1. They found antigen-specific $CD8^+$ T-cell responses directed against all four TAAs in over 50% of the patients. Moreover, survival was significantly increased in patients with TAA-specific $CD8^+$ T-cell response, which suggests that immunotherapy may be beneficial for patients with HCC. Unfortunately, they were unable to expand functional TAA-specific $CD8^+$ T cells from HCC patients *in vitro.*

ENHANCING SPONTANEOUS IMMUNE RESPONSE

Controlling HCC by harnessing naturally occurring, specific immune responses typically fails because the immune responses are not strong enough to overcome the disease. Tumor-specific $CD8^+$ T cells are dysfunctional, regulatory T cells reduce immune response, and tumor cells have acquired mutations, allowing them to escape the immune response. This phenomenon is called cancer immunoediting[38]. Because the immune response is inadequate, it is important to stimulate the immune system and avoid immune escape.

Ablative therapies such as TACE, cryoablation and RFA result in immunogenic tumor cell death by inducing the release of massive amounts of antigens together with "danger signals" from tumor cells, such as damage-associated molecular pattern molecules (DAMPs). This release leads to the activation of DCs and *in vivo* auto-immunization or *in situ* vaccination[39-41]. Increased frequencies of GPC3-specific cytotoxic T cells were observed in patients after RFA and TACE treatment[33]. Hiroishi *et al*[42] studied GPC3, NY-ESO-1- and MAGE-1-specific $CD8^+$ T-cell responses before and after ablative (RFA or TACE) treatment for HCC. They observed that the presence of strong TAA-specific $CD8^+$ T-cell responses suppressed the recurrence of HCC and that the magnitude of a TAA-specific $CD8^+$ T-cell response was a prognostic factor for a prolonged tumor-free interval. Nobuoka *et al*[33] reported that RFA induced a GPC3-specific T-cell response. For the first time, they showed that RFA induced a stronger GPC3-specific immune response than surgical resection because RFA destroys tumor tissue and causes local necrosis followed by the release of tumor-associated antigens, whereas surgery removes almost all of tumor-associated antigens.

From these results, it appears that immunotherapy to induce TAA-specific cytotoxic T lymphocytes after local therapy should be considered for clinical application in patients with HCC.

THERAPEUTIC VACCINATION FOR HCC: DENDRITIC CELL VACCINATION

The aim of cancer vaccination is the induction and perpetuation of a tumor-specific immune response by eliciting effector T cells that can specifically decrease tumor load and induce immunological memory to control tumor relapse. Thus, dendritic cell-based therapies aim to either induce new or enhance pre-existing antigen-specific T cells, but they have no direct effect on tumor cells. Instead, these vaccines affect on different cell types of the immune system, which can induce tumor cell death. Once targetable tumor antigens have been identified, they can be used to load professional antigen presenting cells, *i.e.*, DCs. DCs play key roles in both innate and adaptive immunity[43]. DCs can either be loaded *in vivo* or *ex vivo*. DCs capture antigens and convert them to peptides that are presented on major histocompatibility complex (MHC) molecules, which are recognized by T cells in lymphoid organs. In addition, RFA treatment has been shown to up-regulate tumor antigen expression and

Table 1 Clinical trials of immune therapy in hepatocellular carcinoma

Regimen	Patients, *n*	Clinical response	Reference
HCC vaccines			
DC's + auto-tumor lysate	31	PR: 12.9%, SD: 54.8%	Lee *et al*[53], 2005
DC's + 4 AFP peptides	16	No clinical response	Butterfield *et al*[30], 2007
DC's + HepG2 lysate	25	PR + SD: 28%	Palmer *et al*[52], 2009
GV 1001 + GM-CSF	40	SD: 45.9%	Greten *et al*[71], 2010
GPC3 peptides	33	PR: 3%, SD: 57.6%	Sawada *et al*[72], 2012
DC's	30	PR: 17%, SD: 60%	El Ansary *et al*[49], 2013
Immune checkpoint inhibitors			
Tremelimumab	21	PR: 17.6%, SD: 58.8%	Sangro *et al*[62], 2013

PR: Partial response; SD: Stable disease.

MHC presentation in tumor cells in an *in vivo* mouse model[44]. These results suggest the effectiveness of combing active immunotherapy and conventional therapies, such as RFA, to augment tumor cell recognition by T cells and eventually improve clinical outcome.

However, both infections and tumors can suppress immunity through the release of cytokines, such as interleukin (IL)-6, vascular endothelial growth factor (VEGF) and IL-10, which suppress DC activity. Moreover, tumors may condition local DCs to form suppressive T cells, diminishing immune responses against cancer cells. In humans, immature DCs are capable of inducing antigen-specific regulatory $CD8^+$ T cells[45]. It is therefore imperative to use mature DCs to avoid the immunosuppressive effects of tumor cells[46] or to counteract the inhibitory mechanisms of tumor cells when using anti-PD1/PD-L1, or anti-CTLA4, treatments (discussed in more detail below). Several strategies have been developed to accomplish this, including *ex vivo* DC activation; the addition of strong activation stimuli; the optimization of administered tumor-associated antigens; and the optimization of the dose, frequency and route of administration of a vaccine. Using these strategies, DC vaccines are capable of initiating strong cytotoxic T-lymphocyte responses against TAAs, and DC vaccination remains a good approach for immunotherapy of HCC[47,48].

Ex vivo-matured DCs pulsed with tumor lysate were injected intra-dermally into patients with advanced HCC. This therapy was shown to be safe, and there were significantly more AFP-specific $CD8^+$ T cells 1 mo after DC injection. There were fewer patients with progressive disease in the vaccinated *vs* in the non-vaccinated group, and the mean patient survival was prolonged, although not significantly[49]. In a phase 1 study, 10 patients were treated with radical microwave ablation of HCC followed by 3 courses of mature DC injection into the inguinal lymph nodes and the infusion of immature DCs into microwave-treated HCC lesions. No grade 3/4 toxicity was observed. The percentage of $CD4^+CD25^+$ regulatory T lymphocytes decreased significantly and the percentage of $CD8^+CD28^-$ effector cells increased significantly by 1 mo after therapy, but this encouraging result disappeared by 6 mo after therapy[50].

In another phase 1 study, 5 patients were treated with TACE, followed by repeated DC vaccination. The vaccine was prepared by pulsing DCs with cytoplasmic transduction peptide-attached AFP, GPC3 and MAGE-1 recombinant fusion proteins. Mature DCs were injected subcutaneously near the inguinal lymph nodes in combination with the application of Toll-like receptor 7 agonist at the site of injection. In all patients, the vaccine was safe and elicited TAA-specific T cell responses. In one patient, this resulted in stable disease[51]. Thirty-five patients were included in a phase Ⅱ clinical trial investigating the safety and efficacy of intravenous vaccination with mature autologous DCs pulsed *ex vivo* with tumor cell lysate. Patients received up to 6 vaccines at 3-wk intervals. The treatment was safe and well tolerated, generating antigen-specific immune responses in some cases; unfortunately, there were very low clinical responses[52]. In another phase Ⅱ clinical trial that assessed 31 patients with advanced HCC, DCs were pulsed with autologous tumor lysates. The patients were treated with five courses of DC vaccination intravenously at weekly intervals and in 17 patients this was followed by monthly boost vaccinations. The treatment was safe. Among these 31 patients, 4 had a partial response and 17 had stable disease. Moreover, the patients treated with the boosted therapy had a 1-year survival rate of 63.3% *vs* 10.7% in patients treated with the initial pulsed therapy alone[53]. These results are promising, but the overall results of DC vaccination are unsatisfactory and should be improved (Table 1).

One possibility for improvement could be combining vaccination with anti-angiogenic tyrosine kinase inhibitors (TKIs), such as sorafenib. This strategy targets multiple components of the tumor microenvironment and could mediate an anti-tumor response by immunogenic modulation and immune subset conditioning[54]. Additionally, the vascular changes caused by TKIs affect tumor-infiltrating immune cells, so the combining TKIs with immune therapy could enhance the clinical benefit[55].

Another area of concern is the recurrence of HCC after liver transplantation[56]. Indeed, it has been shown that the recurrence rates of HCC after liver transplantation for HCC are high and have a dismal prognosis[57]. Disease progression was significantly faster in transplanted patients than in patients who underwent surgical resection of HCC, probably due to immunosuppression and reduced host immunity[58]. Immunosuppression *via* mammalian targets of rapamycin seems to decrease recurrence rates and

to slow progression in case of recurrence[59]. DC vaccination has not been tested in liver transplant patients, but it has been shown to be safe and promising with regards to immunological responses after allogeneic-hematopoietic cell transplantation[60].

IMMUNE CHECKPOINT BLOCKADE

The intensity of an immune response results from the balance between stimulatory and inhibitory signals, known as immune checkpoints. These checkpoints are often activated by tumor signals and promote tumor evasion from immunity. Cytotoxic T lymphocyte-associated antigen (CTLA-4) and programmed death 1 (PD-1) are the two most studied immune checkpoints, and inhibitory antibodies against these are already being used in clinical trials. The mechanisms of action of CTLA-4 and PD-1 and their possible roles in treating HCC were recently reviewed[61]. The CTLA-4 inhibitor, tremelimumab was studied in a phase 1 clinical trial. It was well tolerated, and 76% of the enrolled patients had either a partial response or stable disease, of which 45% were stable for more than 6 mo[62]. PD-1 was found in liver-infiltrating lymphocytes and its ligands PD-L1 and PD-L2 were shown to be up-regulated in HCC tissue[63]. Currently, several anti-PD-1 and anti-PD-L1 antibodies are being developed, and their use in clinical studies of HCC is planned.

PERSPECTIVES

Antigen-encoding mRNA is emerging as a particularly promising vaccination tool as it has many advantages to offer. Its advantage over classical vaccination with peptides is that RNA encodes genetic information corresponding to whole antigens. RNA processing by endogenous cell machinery and presentation on MHC complexes are independent of the HLA-subtype of a patient. In addition, RNA does not pose a risk of genomic integration, giving it a favorable safety profile compared to DNA. Due to its transient nature, RNA is only expressed during a controlled period of time and is eventually degraded into natural products. Furthermore, RNA acts as its own adjuvant, prompting co-stimulatory signals, which is advantageous in the context of RNA-based immunotherapy. Even hard-to-modify cells, such as DCs can be modified with mRNA. Two routes for exogenous mRNA delivery into DCs have been applied: either *ex vivo* delivery with subsequent adoptive transfer of transfected DCs or the direct administration of mRNA with subsequent uptake *in vivo*. For the former, DCs derived from patients are cultivated and electroporated with mRNA followed by their restitution into the patient.

In situ modification of DCs by immunization via the direct application of naked mRNA was first described 25 years ago[64]. Since then, several studies have shown anti-tumor immune responses following the injection of naked mRNA in a variety of mouse models[65]. It is assumed that intradermal delivery of mRNA results in its uptake by Langerhans' cells and dermal DCs at the injection site, which are transported to draining lymph nodes. It is moreover assumed that these DCs transfer their antigenic cargo to lymph node-resident $CD8^+$ DCs when they arrive in the draining lymph nodes. Direct injection of mRNA into lymph nodes results in the uptake of mRNA and has been shown to be superior to intradermal injection of mRNA with respect to the induction of antigen-specific T cell responses[65]. Currently, intranodal administration of mRNA is proposed as the optimal route for delivery because lymph nodes harbor a high number of DCs. Intranodal administration creates a microenvironment that favors the induction of potent and sustained immune responses. Lymph node resident dendritic cells up-regulate CD86, and this is required for the efficient activation of naïve T cells and for immunologic memory[66,67].

Currently, we are performing a phase 1 study investigating the feasibility and safety of the intranodal injection of a TriMix-based mRNA vaccine. The concept was studied in melanoma animal models[67]. The idea is to use mRNA for the *in vivo* modification of DCs by direct administration into lymph nodes, which harbor a high number of DCs in close contact with T cells. For a detailed description, we refer to our recent review[68]. In melanoma patients, we previously showed that TriMix mRNA (mRNA encoding CD40 ligand, CD70 and a constitutively active form of TLR4), induced the activation of DCs, resulting in the induction of a T cell attracting and stimulatory environment[69]. Moreover, the co-administration of tumor antigen mRNA and TriMix resulted in the recruitment of antigen-specific $CD4^+$ and $CD8^+$ T cells. Simultaneous delivery of TriMix and antigen mRNA significantly enhanced the induction of antigen-specific T cells compared to intranodal delivery of antigen mRNA alone[67,68]. In the current study, TriMix mRNA and mRNA encoding the target antigens GPC3 and MAGE-C2 mRNA were injected intranodally the same day as RFA treatment of HCC thereby increasing tumor antigen load. The mRNA injection was repeated 3 times at 2-wk intervals. If this intervention appears to be safe, we plan to add immune checkpoint blockers, which could increase the efficacy of the vaccination as we showed in previous melanoma studies[68]. To avoid systemic autoimmune toxicity caused by anti-CTLA-4 or anti-PD-1 treatment, mRNA encoding factors that have the ability to block CTLA-4 or PD-1 can be used[70].

CONCLUSION

Hepatocellular carcinoma is a very common cancer with a high mortality, and the current standard treatment for it is unsatisfactory. Tumor recurrence and the development of new cancer after treatment is frequent and remains a major problem. Because immunotherapy not only treats an existing tumor, but

also has the potential to prevent the development of new cancers in the cirrhotic liver, vaccination for HCC remains an attractive treatment option. In this context, the combination of active specific immunotherapy with ablative therapy might be an appealing and feasible approach and may provide better results than individual treatments. Immunotherapy will be most effective during or shortly after ablative therapy, when tumor cells are dying and an active immune response has commenced. This first "priming" by the ablative therapy should be sustained by "booster" immunizations to maintain immune control over the tumor. One difficulty is the immune tolerance of the liver, and the immunosuppressive environment of cancer, which makes *in situ* activation of immune cells problematic. This problem can be overcome by *ex vivo* activation of DCs or by *in vivo* activation of DCs in skin or lymph nodes. The addition of immune checkpoint inhibitors will undoubtedly add significantly to efficacy, but this will probably increase side effects.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Controversies regarding and perspectives on clinical utility of biomarkers in hepatocellular carcinoma

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Abstract

The prevalence of hepatocellular carcinoma (HCC) worldwide parallels that of persistent infection with the hepatitis B virus (HBV) and/or hepatitis C virus (HCV). According to recommendations by the World Health Organization guidelines for HBV/HCV, alpha-fetoprotein (AFP) testing and abdominal ultrasound should be performed in routine surveillance of HCC every 6 mo for high-risk patients. These examinations have also been recommended worldwide by many other HCC guidelines over the past few decades. In recent years, however, the role of AFP in HCC surveillance and diagnosis has diminished due to advances in imaging modalities. AFP was excluded from the surveillance and/or diagnostic criteria in the HCC guidelines published by the American Association for the Study of Liver Diseases in 2010, the European Association for the Study of the Liver in 2012, and the National Comprehensive Cancer Network in 2014. Other biomarkers, including the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), des-γ-carboxyprothrombin, Dickkopf-1, midkine, and microRNA, are being studied in this regard. Furthermore, increasing attention has focused on the clinical utility of biomarkers as pre-treatment predictors for tumor recurrence and as post-treatment monitors. Serum and tissue-based biomarkers and genomics may aid in the diagnosis of HCC, determination of patient prognosis, and selection of appropriate treatment. However, further studies are needed to better characterize the accuracy and potential role of these approaches in clinical practice.

Key words: Hepatocellular carcinoma; Biomarker; Guideline; Surveillance; Diagnosis; Prognosis

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Core tip: Hepatocellular carcinoma (HCC) is a major global health problem due to the high prevalence

of the risk factors hepatitis B virus and hepatitis C virus infection. Thus, a good surveillance program and diagnostic strategy for the early detection of HCC should be available. This review summarizes the controversies regarding and perspectives on clinical utility of biomarkers in HCC, especially the current role of alpha-fetoprotein and des-γ-carboxyprothrombin. In addition, research frontiers and prospects for novel biomarkers to evaluate the prognosis for HCC and to facilitate post-treatment monitoring are reviewed.

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INTRODUCTION

In 2012, liver cancer was the fifth most common cancer (782000 new cases) and the second leading cause of cancer-related death (746000 cases) worldwide[1]. Hepatocellular carcinoma (HCC) accounts for more than 90% of primary liver cancers and is a major global health problem because of the high prevalence of the risk factors hepatitis B virus (HBV) and hepatitis C virus (HCV) infection[2]. Worldwide, approximately 54% of HCC cases can be attributed to HBV infection, while 31% can be attributed to HCV infection, with the remaining 15% associated with other causes[3-5]. In accordance with the recommendations of the World Health Organization (WHO), vaccination against hepatitis B has been implemented in many countries since 1991[6]. In addition, an enhanced understanding of the pathophysiology of HBV and HCV infection has led to developments in diagnostic procedures and improvements in therapy and prevention, so the clinical care for patients with HBV- or HCV-related liver disease has advanced considerably over the past two decades[7-10]. However, the incidence of HCC worldwide continues to rise, likely due to the often prolonged period between viral infection and manifestation of HCC[2,11,12]. Some studies estimate that up to 20%-30% of patients infected with HBV and/or HCV will develop a progressive liver disease leading to cirrhosis and HCC[13,14]. Cirrhosis rates begin to become significant after 20 years of infection, and HCC rates begin to become significant after 30 years of infection[15,16]. Thus, a good surveillance program and diagnostic strategy for the early detection of HCC should be available.

Serum biomarkers are striking potential tools for surveillance and early diagnosis of HCC thanks to the non-invasive, objective, and reproducible assessments they potentially enable. Worldwide, alpha-fetoprotein (AFP) testing and abdominal ultrasound (US) every 6 mo are recommended for routine surveillance of HCC in high-risk patients according to many HCC guidelines[17]. AFP has also been used as a diagnostic test for HCC and to evaluate prognosis and monitor recurrence following treatment[18]. However, controversy regarding the clinical utility of AFP has arisen in recent years. AFP was excluded from the surveillance and diagnostic criteria in the HCC guidelines published by the American Association for the Study of Liver Diseases (AASLD) in 2010[19], and AFP was not recommended as a sensitive or specific diagnostic test in the HCC guidelines published by the European Association for the Study of the Liver (EASL) in 2012[20] and in the HCC guidelines published by the National Comprehensive Cancer Network (NCCN) in 2014[21]. In Asian countries, AFP was still recommended for HCC surveillance in combination with US and was recommended as an adjunctive diagnostic tool by the HCC guidelines published by the Asian Pacific Association for the Study of the Liver (APASL) in 2010[22], by the current guidelines published in China in 2011[23], and by the current guidelines published in Japan in 2013[24]. Other biomarkers, including the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), des-γ-carboxyprothrombin (DCP), Dickkopf-1 (DKK1), midkine (MDK), and microRNA (miRNA), are being studied in this regard. Furthermore, increasing attention has focused on the clinical utility of biomarkers as pre-treatment predictors for tumor recurrence and as post-treatment monitors.

This article provides an overview of current biomarkers in HCC with respect to their clinical utility in surveillance, early diagnosis, prediction of prognosis, and monitoring of response to therapy. The controversy of using biomarkers in these settings is discussed in light of typical HCC guidelines worldwide, and the prospects for novel HCC biomarkers are also discussed.

HBV/HCV GUIDELINES PROMOTING THE MANAGEMENT OF HCC IN HIGH-RISK PATIENTS

The prevalence of HCC worldwide parallels that of viral hepatitis. Chronic HBV infection is a leading cause of HCC in most African and Asian countries, except Japan, and chronic HCV infection predominantly contributes to HCC in Europe, Japan, and North America[25,26]. An estimated 2 billion people worldwide have signs of past or present infection with HBV, and 240 million people have a chronic infection[7]. More than 185 million people have been infected with HCV, and one third of those will develop a chronic infection[27]. Longitudinal studies of untreated individuals with chronic HBV infection indicate that they have an 8%-20% cumulative risk of developing cirrhosis over 5 years[28,29]. In people with cirrhosis, there is an approximately 20% annual risk of hepatic decompensation and an annual incidence of

HCC of < 1% to 5%[2,30]. In persons with chronic HCV infection, the risk of liver cirrhosis is 15%-30% within 20 years[31,32], and the risk of HCC in persons with cirrhosis is approximately 2%-4% per year[33].

Universal hepatitis B immunization programs that target infants, with the first dose at birth, have been highly effective in reducing the incidence and prevalence of hepatitis B in many countries where infection is endemic[34-37]. However, these programs will not affect HBV-related deaths until several decades after their introduction[38]. Many guidelines focusing on the management of HBV/HCV infection have been published worldwide, such as guidelines published by the AASLD[39,40], the APASL[41,42], the EASL[43,44], the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN)[45], the Canadian Association for the Study of the Liver (CASL)[46], and the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN)[47]. The WHO also published its first guidelines for the prevention, care, and treatment of people living with chronic HCV infection in April 2014[48], and the WHO published similar guidelines for chronic HBV infection in March 2015[38] to help low- and middle-income countries, in particular, to plan for the development and expanded scale of hepatitis B/C prevention, care, and treatment.

According to recommendations in HBV/HCV guidelines from the WHO[38,48], AFP testing and US were suggested to be performed for routine surveillance of HCC every 6 mo in individuals with cirrhosis. In Japan, where HCV is the most significant etiological factor for developing HCC, there is a more detailed definition for high risk patients of HCC - the "very-high-risk group" that includes patients with HBV- or HCV-related liver cirrhosis, and the "high-risk group" that includes patients with HBV- or HCV-related chronic liver disease or liver cirrhosis due to other causes[49].

BIOMARKERS IN HCC SURVEILLANCE: CONTROVERSIES AND PROSPECTS

Surveillance of patients at increased risk for HCC has been shown to result in the detection of early-stage tumors and an increased likelihood of undergoing potentially curative therapies[50-54]. The overall 5-year survival rate for patients with HCC is about 40%, but liver resection of early HCC could result in a 5-year survival rate of 60%-70%[55-57]. Surveillance is therefore required to detect HCC at an early stage and increase the chances of effective treatment.

AFP as a traditional biomarker for HCC surveillance: Its current role and controversy

AFP testing and US are the most widely used methods of HCC surveillance[58,59]. Data have indicated that AFP testing and US every 6 mo affect disease-specific mortality compared to no intervention [odds ratio: 0.57, 95% confidence interval (CI): 0.37-0.89][38]. In addition, surveillance every 6 mo using both AFP and US has been found to be the most cost-effective strategy[60-62].

AFP has been widely used in clinical practice as a traditional biomarker for HCC surveillance over the past two decades[63-65]. However, there is increasing debate regarding the utility of AFP as a surveillance test[66-68]. Analysis of recent studies has indicated that AFP testing lacks adequate sensitivity and specificity for effective surveillance[69-71]. AFP levels are normal in up to 40% of patients with HCC, particularly during the early stage of the disease (low sensitivity)[72-74]. Elevated AFP levels may be seen in patients with cirrhosis or exacerbation of chronic hepatitis or cholangiocarcinoma (low specificity)[75,76]. In addition, some studies have indicated that AFP has substantially limited diagnostic accuracy in detecting small HCC[77].

Given these findings, US is regarded as a more appropriate test for surveillance with an acceptable diagnostic accuracy (sensitivity ranging from 58% to 89%, specificity greater than 90%)[69,78]. Currently, US is recommended as the only tool for HCC surveillance in some Western countries. AFP was excluded from the surveillance criteria in the HCC guidelines published by the AASLD in 2010[19], and AFP is regarded as a suboptimal tool for surveillance according to the HCC guidelines published by the EASL in 2012[20]. Nevertheless, the performance of US in early detection of HCC is highly dependent on the expertise of the examiner and the quality of the equipment. A randomized controlled trial found that surveillance with AFP in conjunction with US reduced the mortality of HCC[79], and the position that AFP should be included in the HCC surveillance guidelines of the AASLD is gaining support[80]. Currently, the combination of AFP and US at approximately 6 mo intervals is still recommended by many HCC guidelines in Asia, such as guidelines in Japan[24], China[23], and guidelines published by the APASL[22] (Figure 1). Thus, whether AFP should be excluded from surveillance criteria needs to be investigated in more large, randomized controlled trials.

The combined testing of AFP, AFP-L3, and DCP for HCC surveillance

The effectiveness of surveillance depends on various factors. Inclusion of new diagnostic tests in surveillance programs may allow the detection of additional small HCC. Two other serum biomarkers besides AFP - the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and des-γ-carboxyprothrombin (DCP, also known as prothrombin-induced by vitamin K absence-Ⅱ, PIVKA-Ⅱ) - have been studied around the world to explore their clinical usefulness in determining the risk of HCC in high-risk populations.

The clinical utility of highly sensitive AFP-L3 in early prediction of HCC developing in patients with chronic

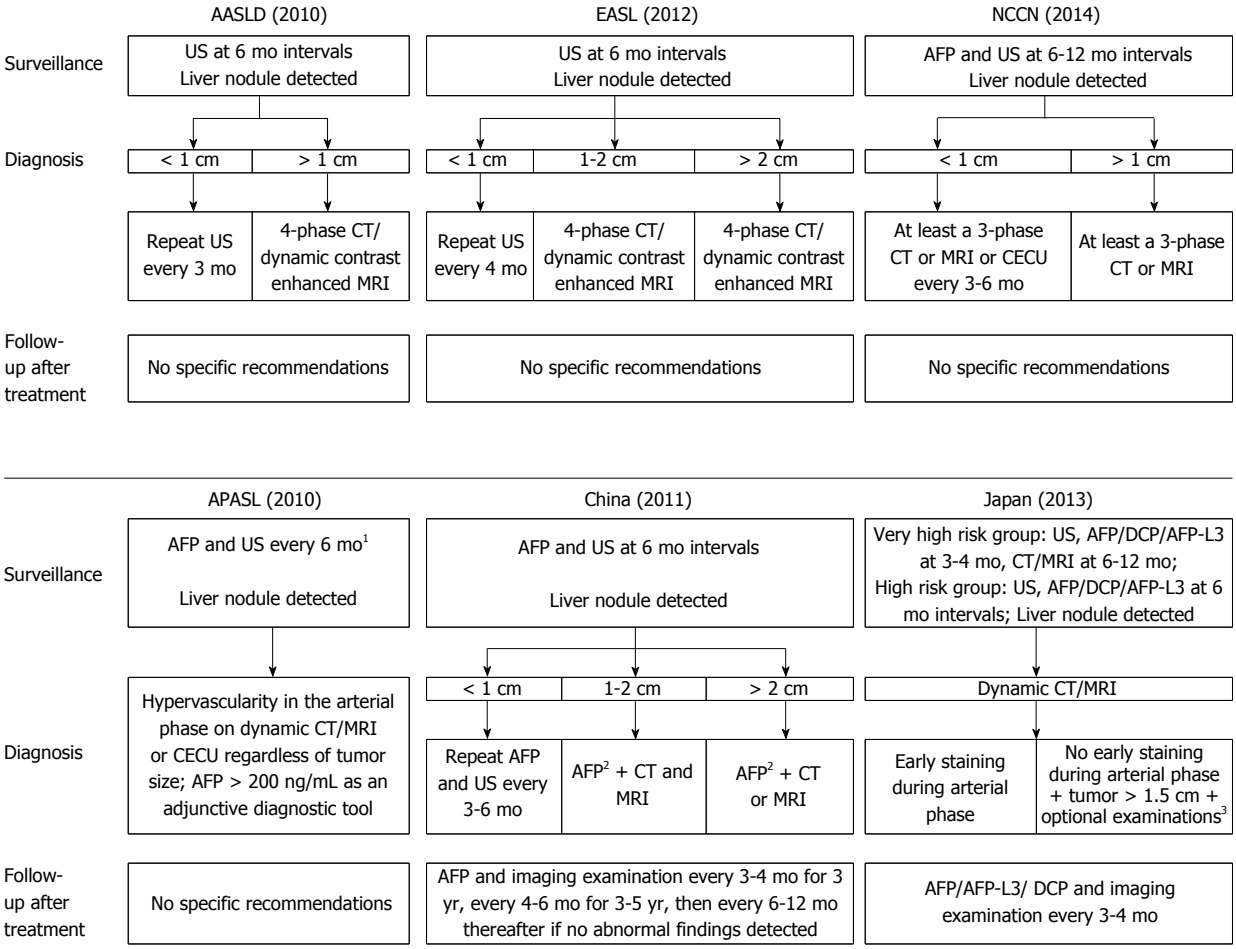

Figure 1 The clinical utility of biomarkers according to typical hepatocellular carcinoma guidelines worldwide. A: Typical hepatocellular carcinoma (HCC) guidelines in Western countries; B: Typical HCC guidelines in Asian countries. [1]Alpha-fetoprotein (AFP) alone is not recommended for diagnosis of HCC; the measurement of both AFP and des-γ-carboxyprothrombin (DCP) provides a higher level of sensitivity without decreasing specificity; [2]AFP ⩾ 400 ng/mL over 1 mo or AFP ⩾ 200 ng/mL over 2 mo; [3]Optional examinations include computed tomography (CT)-angiography, liver-specific contrast-enhanced magnetic resonance imaging (MRI), contrast ultrasound (US), or liver tumor biopsy.

HBV or HCV infection was recently evaluated in a large Japanese study, and results indicated that elevated AFP-L3 was an early predictor of HCC development even if AFP levels were low and suspicious US findings were absent. Elevated AFP-L3 was noted in 34.3% of patients 1 year prior to diagnosis of HCC[81]. Numerous studies have found that the combined testing of DCP and AFP has a sensitivity of 47.5%-94.0% and a specificity of 53.3%-98.5% in early detection of HCC, and these figures are higher than those for either marker alone[82-84]. In some countries such as Japan, combined measurement of DCP and AFP-L3 reportedly increased the detectability of small HCC[85,86].

There are also several biomarkers in addition to AFP, AFP-L3, and DCP, such as Golgi protein 73 (GP73)[87,88], interleukin-6 (IL-6)[89,90], and squamous cell carcinoma antigen (SCCA)[91], that are currently being studied. However, these biomarkers have usually been evaluated, alone or in combination, in a diagnostic rather than surveillance setting. Moreover, their diagnostic performance has often been assessed with a markedly higher prevalence of HCC than would be expected in the context of surveillance.

Current expert opinion from Western countries has been rather critical of the clinical value of biomarkers[92]. Imaging-based surveillance criteria were recommended in guidelines from Western countries, such as the updated HCC guidelines published by the AASLD in 2000[19] and similar guidelines published by the EASL in 2012[20]. In Asian countries, as typified by HCC guidelines in Japan, US and measurement of AFP, AFP-L3, or DCP are recommended to be performed at intervals of 3-4 mo for the very-high-risk group (patients with HBV- or HCV-related liver cirrhosis) and at 6 mo intervals for the high-risk group (patients with HBV- or HCV-related chronic liver disease or liver cirrhosis due to other causes)[24,93].

BIOMARKERS FOR DIAGNOSIS OF HCC: THEIR EVOLUTION AND PROSPECTS

Accurate diagnosis of small liver nodules is of paramount importance. In general, the tests used to

diagnose HCC around the world include diagnostic imaging, serological diagnosis, and histological diagnosis. Prior to 2000, a definite diagnosis was based on a biopsy, but this approach had several limitations related to feasibility due to location and risk of complications, such as bleeding or needle-track seeding[94]. With the development of imaging techniques, a unique dynamic radiological behavior - contrast uptake in the arterial phase of computed tomography (CT), magnetic resonance imaging (MRI), angiography, or US - represented the backbone of radiological diagnosis of early HCC.

The role of AFP in diagnosis of HCC: No longer in use or used as an adjunctive diagnostic tool?

AFP has served as a diagnostic test for HCC since the 1970s, when most patients with HCC were diagnosed at an advanced stage and with clinical symptoms. At that time, a level of 500 ng/mL AFP was considered diagnostic[95]. However, the usefulness of AFP as a diagnostic test in small HCC is limited. According to a systematic review, AFP with a cut-off value of 20 ng/mL had a sensitivity, specificity, and a positive likelihood ratio (LR^+) of diagnosing HCC smaller than 5 cm in diameter of 49%-71%, 49%-86%, and 1.28-4.03, respectively; AFP with a cut-off value of 200 ng/mL had a sensitivity, specificity, and an LR^+ of 4%-31%, 76%-100%, and 1.13-54.25, respectively[77].

Although the sensitivity and specificity of serum AFP as a biomarker is being challenged, a high level or a steadily increasing level of serum AFP strongly suggest development of HCC[96,97]. Elevated serum AFP and a typical enhancement pattern in dynamic imaging have provided critical clues for the diagnosis of HCC over the past few decades. Nevertheless, the importance in AFP has diminished in recent guidelines for diagnosis of HCC, and the importance of imaging has increased based on the high accuracy of up-to-date radiologic modalities[98].

According to the diagnostic criteria in the HCC guidelines published by the EASL in 2000[99] and similar guidelines published by the AASLD in 2005[25] the NCCN in 2009[26], HCC diagnosis is based on the tumor size, AFP, and imaging examination. Guidelines from the Korean Liver Cancer Study Group (KLCSG) published in 2003[100] also featured algorithms similar to those in the aforementioned guidelines, with the exception that HCC was diagnosed based on imaging and AFP, regardless of tumor size. However, the updated HCC guidelines published by the KLCSG in 2009 suggested that a tumor of 2 cm or larger in patients with liver cirrhosis that has characteristics typical of HCC in dynamic contrast enhancement CT or MRI could be diagnosed as HCC regardless of the serum AFP level[101]. According to updated HCC guidelines published by the AASLD in 2010, nodules larger than 1 cm found during US surveillance of a cirrhotic liver should be investigated further with either a four-phase multidetector CT scan or dynamic contrast enhanced MRI. If the appearance of the nodule is typical of HCC, the lesion should be treated as HCC; if the findings are not characteristic or the vascular profile is not typical, a second contrast enhanced study involving another imaging modality should be performed, or the lesion should be biopsied[19]. In agreement with updated guidelines from the AASLD, the panel that drafted the HCC guidelines of the NCCN in 2014 also considered an imaging finding of classic enhancement to be more definitive in this instance, since the level of serum AFP may be elevated in persons with certain nonmalignant conditions, or it may be within normal limits in a substantial percentage of patients with HCC[21]. According to the HCC guidelines published by the APASL in 2010[22], typical HCC can be diagnosed based on imaging regardless of tumor size if a typical vascular pattern (*i.e.*, arterial enhancement with portal venous washout) is obtained on dynamic CT/MRI or contrast-enhanced US. According to the same guidelines, AFP was recommended as an adjunctive diagnostic tool, and AFP alone was not recommended for diagnosis of HCC. Similar recommendations were made by HCC guidelines published in China[23] and Japan[24].

Perspectives on the combined testing of AFP and other biomarkers for diagnosis of HCC

Advances in technology and an increased understanding of HCC biology have led to the discovery of novel biomarkers. To date, many biomarkers have been proposed as a complement or substitute for AFP in the diagnosis of HCC. AFP-L3 can differentiate an increase in AFP due to HCC from that in patients with benign liver disease[102,103]. AFP-L3 with a cut-off value of 10% had a sensitivity, specificity, and LR^+ in diagnosing HCC smaller than 5 cm in diameter of 22%-33%, 93%-94%, and 4.6-0.8, respectively; AFP-L3 with a cut-off value of 15% had a sensitivity, specificity, and LR^+ of 21%-49%, 94%-100%, and 8.1-45.1 respectively[77]. DCP has also been recognized as a highly specific marker for HCC[104]. DCP with a cut-off value of 40 mAU/mL had a sensitivity, specificity, and LR^+ in diagnosing HCC smaller than 5 cm in diameter of 14%-54%, 95%-99%, and 6.9-29.7, respectively; DCP with a cut-off value of 100 mAU/mL had a sensitivity, specificity, and LR^+ of 7%-56%, 72%-100%, and 3.6-13.0, respectively[77].

Data have indicated that the combined testing of DCP and AFP or AFP-L3 could help to increase the sensitivity of HCC diagnosis[105-107], but this approach is used in only a few countries[17], such as Japan[108,109]. In 2014, a large-scale, multi-center study investigated the measurement of both AFP and DCP in differentiating Chinese patients with HCC (71.18% with HBV infection) from patients without HCC and normal subjects. Results showed that the combined testing of

DCP with a cut-off value of 86 mAU/mL and AFP with a cut-off value of 21 ng/mL resulted in a sensitivity of approximately 90% in diagnosis of HCC, which was significantly higher than that for DCP or AFP alone. This finding held even for a tumor smaller than 2.0 cm[110]. These results suggest that the measurement of both AFP and DCP may facilitate the diagnosis of patients with a broad range of HCC. However, the clinical utility of DCP in China has not been noted by HCC guidelines in China[111,112], and more large-scale prospective studies should be performed to provide sufficient evidence.

In recent years, numerous studies have investigated the clinical usefulness of other biomarkers in the early diagnosis of HCC, including GP73[87,88], glypican-3 (GPC3)[113,114], osteopontin[115,116], and vascular endothelial growth factor (VEGF)[117]. Most recently, research on DKK1 and MDK as diagnostic serum biomarkers has garnered interest. In 2012, Shen *et al*[118] published a retrospective, cross-sectional study involving 424 patients with HCC and 407 controls without HCC, and they found that DKK1 was highly accurate at diagnosing AFP-negative patients with HCC, including patients with early-stage HCC. They also found that the measurement of DKK1 and AFP together improved the accuracy with which HCC was diagnosed in comparison to any test alone. In 2013, Zhu *et al*[119] published a study involving 388 patients with HCC and 545 different controls, and they found that serum MDK had a markedly higher level of sensitivity than AFP (86.9% *vs* 51.9%) even when diagnosing very early-stage HCC (80% *vs* 40%). Zhu *et al*[119] also found that MDK could have a sensitivity as high as 89.2% when diagnosing cases of AFP-negative HCC.

Noncoding RNA and miRNA in particular have received considerable attention as novel potential biomarkers over the past few years[120]. Li *et al*[121] found that three miRNAs (miR-25, miR-375, and let-7f) had a sensitivity and specificity as high as 97.9% and 99.1%, respectively, in diagnosing HCC. Zhou *et al*[122] found that a panel of seven microRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) could provide a high level of diagnostic accuracy for identification of HBV-related HCC. Tomimaru *et al*[123] found that the combination of miRNA-21 with AFP improved the power of differentiation between HCC and chronic hepatitis, with a sensitivity of 81.0% and a specificity of 80%. However, the potential for miRNA to serve as a biomarker has not been equally analyzed in all conditions potentially leading to HCC. Systemic analyses of alcoholism, non-alcoholic steatohepatitis (NASH), and HCV-related conditions are pending.

BIOMARKERS: PREDICTION OF PROGNOSIS AND MONITORING OF THE RESPONSE TO THERAPY

Tumor invasiveness, metastasis, and recurrence often result in poor clinical outcomes for patients with HCC[124,125]. Currently, the measurement of biomarker levels both before and after HCC treatment is clinically valuable as a simple way to monitor treatment outcomes (usually in combination with radiological analysis) and to predict prognosis, recurrence, and survival.

AFP, AFP-L3, and DCP: Diagnostic biomarkers could also be used to determine the prognosis for HCC and to facilitate post-treatment monitoring

Several biomarkers that have been evaluated for their power in diagnosing HCC have also been studied for their prognostic significance. A high level of AFP expression in serum correlates with a profound cell proliferation, profound angiogenesis, and limited apoptosis and is associated with a poor prognosis[126,127]. AFP was one of the most robust predictors of death in patients with cirrhosis and HCC[128], and it also has significance at predicting survival after liver transplantation[129]. Changes in AFP while on the waitlist also predicted post-transplant survival, and identifying these changes could facilitate better patient selection to optimize organ allocation and post-transplant outcomes[18]. A change in AFP levels has been found to correlate with radiologic response and overall survival after locoregional therapy. For example, a 50% decrease in AFP levels resulted in a better time-to-progression [hazard ratio (HR): 2.8, 95%CI: 1.5-5.1)] and overall survival (HR: 2.7, 95%CI: 1.6-4.6) in comparison to patients whose AFP levels failed to respond to treatment with transarterial chemoembolization (TACE) or transarterial radioembolization (TARE)[130]. Whether AFP is useful at predicting the response to sorafenib is controversial[127,131], and several studies have indicated that AFP response was correlated with time-to-progression (7.9 mo *vs* 2.4 mo, $P = 0.004$) and overall survival (13.3 mo *vs* 8.2 mo, $P = 0.022$)[132].

AFP-L3[133,134] and DCP[135,136] were also identified as prognostic biomarkers for survival after resection of HCC. Patients that have undergone resection of HCC and who had elevated levels of AFP, AFP-L3, and DCP at the baseline had a worse prognosis than patients who tested positive for just one or two of the markers before surgery[137-140].

Among the current guidelines for HCC management worldwide, the guidelines of the NCCN[21] published in 2014 recommend high-sectional imaging every 3-6 mo for 2 years and then every 6-12 mo for post-treatment monitoring. If AFP levels are initially elevated, the guidelines recommend that monitoring be performed every 3 mo for 2 years and then every 6-12 mo. The Indian National Association for Study of the Liver (INASL) published the first guidelines in India in 2014[141], and these guidelines make similar recommendations. The guidelines recommend that post-treatment monitoring be performed with dynamic CT or MRI studies every 3 mo for the first 2 years and then routine surveillance every 6 mo thereafter. The

guidelines also note that the serum tumor markers AFP and DCP may help to evaluate the response to treatment or evaluate follow-up when AFP or DCP is elevated at diagnosis and when AFP or DCP decreases after treatment but rises again. The guidelines do note, however, that tumor markers cannot replace imaging modalities. According to the HCC guidelines published in Japan in 2013[24], follow-up using the serum biomarkers AFP, AFP-L3, and DCP and imaging should be performed every 3-4 mo after treatment. According to HCC guidelines published in China in 2011[23], post-treatment monitoring with AFP and imaging should be performed every 3-4 mo for 3 years, every 4-6 mo for 3-5 years, and then every 6-12 mo thereafter if no abnormal findings are detected.

Research frontiers and prospects for novel biomarkers to evaluate the prognosis for HCC and to facilitate post-treatment monitoring

Biomarkers, including DKK1[142], GPC3[143], and indo-cyanine green 15 min after administration (ICG-R15)[144], reflect current knowledge of the pathways involved in hepatocarcinogenesis and appear to have prognostic value. However, prospective validation studies still need to be performed. In patients with advanced HCC who are treated with sorafenib, serum VEGF and angiopoietin 2 (Ang2) levels were identified as independent prognostic factors for overall survival[127].

Moreover, gamma-glutamyl transpeptidase (GGT) was identified as a prognostic maker by studies of different subgroups of patients published over the past 5 years[145]. Sheen *et al*[146] found that patients who had HCC with type B GGT mRNA had worse outcomes, earlier recurrence, and more post-recurrence deaths. Several studies of patients with HCC undergoing hepatic resection have revealed a correlation between elevated levels of GGT and worse survival for patients with HBV-related HCC, Child-Pugh A liver function, or multi-nodular tumors[147-149]. In addition, several studies have also revealed the predictive value of GGT in patients with unresectable HCC who were treated with TACE or chemotherapy[150-153].

In addition to their diagnostic potential, miRNAs may help to predict prognosis for HCC. Tomimaru *et al*[123] found that the level of miR-21 expression was high in Asian patients with HCC and that the level declined after surgery. They also found that a high level of miR-21 expression in plasma correlated with a shorter cumulative survival following treatment. Köberle *et al*[154] found in European patients with HCC that higher levels of miR-1 and miR-122 expression were associated with longer overall survival compared to lower levels of expression of those miRNAs. They concluded that miR-1 may be a predictive biomarker of HCC independent of liver function. A 31-miRNA signature correlates with the stage of disease, and a distinct 20-miRNA signature that is associated with metastasis of HCC has also been identified[155]. These findings constitute mounting evidence that miRNA signature profiling can be of use in prognostic stratification. Despite their promising potential, miRNA-based biomarkers pose several problems in terms of their use in clinical practice[156].

CONCLUSION

Current HCC guidelines in Western countries have been rather critical of the clinical value of biomarkers. Over the past few decades, a simple approach in the form of measuring AFP levels has been widely used for routine surveillance and noninvasive diagnosis of HCC and to evaluate prognosis and monitor recurrence after treatment. AFP was excluded from the surveillance and/or diagnostic criteria in HCC guidelines published by the AASLD in 2010, the HCC guidelines published by the EASL in 2012, and the HCC guidelines published by the NCCN in 2014. Nonetheless, AFP is still regarded as a useful surveillance tool and an adjunctive tool by many HCC guidelines in Asia, such as guidelines from Japan, guidelines from China, and guidelines published by the APASL.

Advances in technology and an increased understanding of HCC biology have led to the discovery of novel biomarkers. Data have indicated that the combined testing of AFP, AFP-L3, and DCP could help to increase the sensitivity of diagnosis of HCC, but this approach is currently used in only a few countries, such as Japan. In recent years, numerous studies have investigated the clinical usefulness of some novel biomarkers in early diagnosis of HCC, including GP73, GPC3, osteopontin, VEGF, DKK1, MDK, and miRNA. Moreover, the prognostic significance of these biomarkers has also been evaluated. Serum and tissue-based biomarkers and genomics may aid in diagnosis of HCC, determination of patient prognosis, and selection of appropriate treatment. However, further studies are needed to better characterize the accuracy and potential role of these approaches in clinical practice. The prevailing hope is that novel biomarkers can support clinicians in their daily practice and improve care for patients with HCC.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Glypican-3 is a prognostic factor and an immunotherapeutic target in hepatocellular carcinoma

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Abstract

Glypican-3 (GPC3) is a cell surface oncofetal proteoglycan that is anchored by glycosylphosphatidylinositol. Whereas GPC3 is abundant in fetal liver, its expression is hardly detectable in adult liver. Importantly, GPC3 is overexpressed in hepatocellular carcinoma (HCC), and several immunohistochemical studies reported that overexpression predicts a poorer prognosis for HCC patients. Therefore, GPC3 would serve as a useful molecular marker for HCC diagnosis and also as a target for therapeutic intervention in HCC. Indeed, some immunotherapy protocols targeting GPC3 are under investigations; those include humanized anti-GPC3 cytotoxic antibody, peptide vaccine and immunotoxin therapies. When considering the clinical requirements for GPC3-targeting therapy, companion diagnostics to select the appropriate HCC patients are critical, and both immunohistochemical analysis of tissue sections and measurement of serum GPC3 level have been suggested for this purpose. This review summarizes current knowledge regarding the clinical implication of GPC3 detection and targeting in the management of patients with HCC.

Key words: Glypican-3; Enzyme-linked immunosorbent assay; Hepatocellular carcinoma; Prognosis; Companion diagnostics; Immunohistochemistry

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Core tip: Glypican-3 is frequently overexpressed in hepatocellular carcinoma (HCC). Accumulating evidence indicates that high glypican-3 expression is a significant prognostic factor that predicts poor outcome of patients with HCC. Thus, it serves as a promising molecular target for the development of novel therapies for HCC, and preclinical and clinical trials targeting glypican-3 are currently underway. Evaluation of the glypican-3 levels

in HCC tissues or in sera of patients with HCC would be of value for predicting the patients' prognosis and companion diagnostics for future glypican-3-targeting therapies.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and is the fifth most common malignant neoplasm worldwide[1]. Despite progress in surgical and non-surgical therapies, the prognosis of HCC remains poor. Although the multi-kinase inhibitor sorafenib prolonged median survival and the time to progression by nearly 3 mo[2], new biomarkers and molecular targets are urgently needed to develop novel treatment strategies.

Glypican-3 (GPC3) is a member of the heparan sulfate (HS) proteoglycan family. It attaches to cell membranes by a glycosylphosphatidylinositol (GPI) anchor[3,4]. GPC3 is widely expressed in human embryos, and it regulates morphogenesis or growth, possibly through insulin-like growth factor, bone morphogenic protein, fibroblast growth factor (FGF) or hedgehog signaling[5-7]. *GPC3* gene mutation results in Simpson-Golabi-Behmel syndrome (SGBS), in which patients display fetal macrosomia and continue to grow and gain weight at an unusual rate with a varying range of dysmorphisms[8,9]. In fact, GPC3-deficient mice exhibit several of the clinical features observed in SGBS, including developmental overgrowth, perinatal death, cystic and dysplastic kidney and abnormal lung development[10]. In the liver, normal expression of GPC3 was identified from gestational weeks 18 to 30, and no GPC3 expression was observed in any normal adult liver tissue[5,11,12]. On the other hand, significantly high levels of GPC3 are expressed in HCC cells compared to normal liver and non-neoplastic liver lesions[11,12]. Therefore, GPC3 is a promising tumor marker and may be a potential molecular target for the development of innovative therapies for HCC[3]. This review focuses on the expression of GPC3 and discusses the possible usefulness of GPC3 as a prognostic marker and an immune-therapeutic target for patients with HCC.

GPC3

In 1988, Filmus *et al*[13] isolated a developmentally regulated cDNA clone, called OCI-5, from a rat small intestine cell line. As the *OCI-5* gene encoded a protein highly homologous to the glypican family, the human gene was renamed *GPC3*[14], and it was found to be located on the human X chromosome (Xp26)[15]. The glypican family consists of six members (GPC1 - GPC6), all of which have a cysteine-rich repeat domain at similar positions[4]. GPC3 is abundantly expressed in the placenta and fetal tissues such as liver, lung and kidney; however, its expression is significantly reduced in adult organs[4]. The *GPC3* gene encodes 580 amino acids that produce a core protein with a mass of 70 kDa. After cleavage between Arg^{358} and Cys^{359} by furin, two subunits linked by disulfide bonds (a 40-kDa N-terminal subunit and a 30-kDa C-terminal subunit) are generated[16]. The mature GPC3 heterodimer is expressed on the cellular surface as a GPI-anchored protein with two HS chains attached to the C-terminal region close to the cell membrane (Figure 1)[3,4]. GPC3 can be released from the cell surface into the extracellular environment. Several forms of secreted GPC3, such as glycated forms with a molecular weight larger than 100 kDa or a 50 kDa fragment lacking HS side chain have been reported[11,17,18]. Therefore, several mechanisms may be involved in the shedding of GPC3. One such mechanism is mediated by notum, a kind of lipase that cleaves GPI-anchored proteins, and it results in the release of the full-length glycated form of GPC3[19]. As shorter forms of soluble GPC3 can be detected in culture supernatant of human HCC cells, an alternative shedding or cleaving enzyme may also be present, area requiring further analysis.

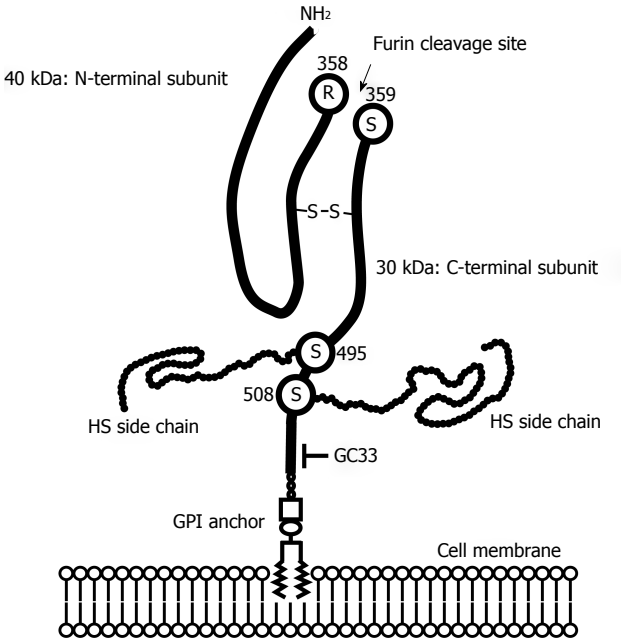

Figure 1 A schematic drawing of the structure of the glypican-3 molecule. The core protein consists of 580 amino acids, and two heparan sulfate (HS) side chains are attached close to the C-terminal portion. Cleavage by furin between Arg^{358} and Cys^{359} results in a 40-kDa N-terminal subunit and a 30-kDa C-terminal subunit linked by a disulfide bond. Monoclonal antibody GC33 recognizes an epitope at the C-terminal portion.

ENHANCED GPC3 EXPRESSION IN HCC TISSUES

In 1997, Hsu *et al*[20] identified an mRNA (MXR7) that was highly expressed in HCC tissue, and it was identical to GPC3 mRNA. The mRNA was hardly detectable in adult non-neoplastic liver (3.2%) but was overexpressed in most HCC tissues (74.8%). They also showed a close correlation between the *GPC3* mRNA level and elevated serum alpha-fetoprotein (AFP) level[20]. Since then, a number of studies have analyzed GPC3 immunohistochemistry (IHC), and the results indicated specific and enhanced expression of GPC3 in HCC tissues[3,11,12,21]. Many IHC studies used the anti-GPC3 mouse monoclonal antibody 1G12 that recognizes a C-terminal portion of GPC3 near its membrane-bound site[11]. One study used two monoclonal antibodies (A1836A and GPC3-C02) that recognized N-terminal and C-terminal portions of GPC-3, respectively, and showed similar immunostaining patterns[4]. Evidence obtained by those IHC studies revealed acceptable specificity and sensitivity of GPC3 IHC for diagnostic purposes in HCC management. While GPC3 was undetectable in normal adult liver, 70%-100% of HCC cases were positive with enhanced immunoreactivity in less-differentiated tumors[3,11,12,21]. Dysplastic regenerative nodules in cirrhotic liver also showed weak and focal immunoreactivity; however, GPC3 was hardly detectable in hepatocellular adenoma and intrahepatic cholangiocarcinoma[11,12,21]. It should be noted that some extrahepatic cancers with known AFP expression, such as yolk-sac tumor, hepatoid adenocarcinoma and other AFP-producing digestive tract cancer variants, also showed high GPC3 expression[22-29], indicating that GPC3 may be an oncofetal protein like AFP. Indeed, evidence suggested that both the *GPC3* and the *AFP* genes may be regulated by a similar transcription factor[30]. In addition, recent IHC studies have suggested that GPC3 can be expressed in other tumors, including thyroid cancers and ovarian clear cell carcinoma[29,31,32].

Recently, another mouse monoclonal antibody (GC33 and its humanized version) was developed, and it recognized an epitope similar to that of 1G12[33]. Notably, GC33 showed a significant cytotoxic activity mediated by antigen-dependent cell cytotoxicity (ADCC) and complement-dependent cell cytotoxicity (CDC) on GPC3-expressing cells[33,34]. Therefore, the humanized GC33 would have significant implications in the development of GPC3-targeting immunotherapy. To develop GPC3-targeting therapies for HCC, it would be necessary to evaluate the GPC3 expression level in individual patients (*i.e.*, companion diagnostics). In formalin-fixed paraffin-embedded tissue sections, anti-GC33 antibody detected HCC cells with a sensitivity and specificity similar to 1G12[35,36]. Four distinct patterns of GPC3 immuno-localization were observed in HCC cells: peri-canalicular membranous, luminal membranous, circumferential membranous and intracytoplasmic[21,36]. Considering the targeting of GPC3 by humanized GC33, it is reasonable to postulate that the circumferential membranous expression of GPC3 in HCC cells is particularly important. Therefore, an IHC scoring system that placed particular emphasis on circumferential membranous immunoreactivity was proposed in the GPC3 IHC study using anti-GC33 antibody[36].

PROGNOSTIC SIGNIFICANCE OF GPC3 IHC

After the diagnostic utility of GPC3 IHC was established in HCC histopathology, extensive studies searching for the prognostic significance of GPC3 expression were conducted in patients with HCC. The clinicopathological studies with GPC3 IHC revealed that higher expression of GPC3 in HCC cells was corelated to a poorer prognosis for patients after curative partial hepatectomy[36,37], and subsequent studies supported this trend[38-41]. The circumferential membranous immunoreactivity scoring system may be superior in predicting the patients' prognosis than a scoring system simply reflecting the positive area ratio[36]. Subsequently, meta-analytic studies of the prognostic significance of GPC3 expression were published. They confirmed that a strong GPC3 IHC score was of prognostic value as it was correlated with shorter overall survival (OS) and disease-free survival (DFS) of HCC patients[42,43]. Therefore, these patients may potentially benefit from adjuvant therapy, particularly that targeting GPC3.

In addition, these IHC studies revealed significant intra-tumoral heterogeneity of GPC3 expression levels in HCC tissue, casting doubt on the usefulness of needle biopsy specimens for the evaluation of GPC3 expression in HCC[21,36,44]. The immunoreactivity observed in a small needle biopsy specimen may not represent the overall level of GPC3 expression of the tumor. This may be a critical issue for needle biopsy specimens if one attempts to use GPC3 IHC as a biomarker of HCC.

SERUM GPC3 LEVEL AS A PROGNOSTIC MARKER OF HCC

As mentioned earlier, GPC3 can be released from the cell surface, and soluble GPC3 is detectable as serum GPC3 (sGPC3). Therefore, measuring sGPC3 levels may be a promising alternative for the estimation of GPC3 expression level in HCC tissue. Indeed, there have been several reports that attempted to measure sGPC3 by enzyme-linked immunosorbent assay (ELISA) in patients with HCC or other chronic liver diseases[11,17,45-54]. The details of each study are

Table 1 Reported enzyme-linked immunosorbent assay studies of serum glypican-3

Ref.	Epitope (AA)[1]	sGPC3 (ng/mL), median (range) *n* (mean ± SD)		
		Healthy	CH/LC	HCC
Capurro *et al*[11] 2003	C-terminal subunit (511-580)	ND (53)	ND/0 (0-117) (18/20) (ND/5.85 ± 26.16)	167.5 (0-2924) (34) (441 ± 669.8)
Hippo *et al*[17] 2004	N-terminal subunit (25-358)	- (96) (0.65 ± 0.32)	- (38) (1.09 ± 0.74)	- (69) (4.84 ± 8.91)
Beale *et al*[46] 2008	ELISA kit (BioMosaics, Burlington, VT)	-	- (41) (125.41 ± 281.05)	- (50) (161.41 ± 422.33)
Tangkijvanich *et al*[47] 2009	C-terminal subunit (511-580)	ND (40)	0 (0-43.6) (100) (-)	46.3 (0-7826.6) (100) (-)
Liu *et al*[48] 2010	ELISA kit (BioMosaics)	-	- (32) (3 cases are > 300 ng/mL)	- (37) (16 cases are > 300 ng/mL)
Yasuda *et al*[49] 2010	ELISA kit (BioMosaics)	-	1.16 (200) (-)	0.92 (200) (-)
Ozkan *et al*[50] 2011	ELISA kit (Wuhan EIAab Science, Wuhan, China)	0.004 (0.004 - 0.008) (28) (-)	0.006 (0.004 - 0.24) (55) (-)	0.005 (0.004-0.09) (75) (-)
Qiao *et al*[51] 2003	ELISA kit (USCN Life Science, Wuhan, China)	- (30) (5.93 ± 5.46)	- (18/40) (9.98 ± 9.60/12.09 ± 9.69)	- (101) (29.29 ± 17.34)
Chen *et al*[52] 2013	N-terminal subunit (350-364)	0 (0 - 563.2) (136) (4.14 ± 31.65)	0 (0-563.2)/6 (0-365.7) (180/124) (10.45 ± 46.02/19.44 ± 50.88)	15.11 (0-2400) (155) (99.94 ± 267.2)
Lee *et al*[53] 2014	ELISA kit (Cusabio Biotech, Wuhan, China)	-	66.4 (40) (-)	75.8 (21.7-482.5) (120) (-)
Abd El Gawad *et al*[54] 2014	ELISA kit (USCN Life Science)	0.99 (0.86-1.67) (10) (-)	2.74 (1.99 - 5.93) (10) (-)	7.7 (4.9-11) (40) (-)
Haruyama *et al*[56] 2015	N-terminal subunit (321-350)	0.12 (0.04 - 0.17) (25) (0.11 ± 0.04)	0.11 (9) (0.12 ± 0.60)	0.24 (0.05-2.96) (115) (0.41 ± 0.51)

[1]Amino acid number recognized. ND: Not detectable; CH: Chronic hepatitis; LC: Liver cirrhosis; sGPC3: Serum glypican-3.

displayed in Table 1. However, the reported values of sGPC3 differed considerably between the studies even in healthy controls. This is presumably because of the different antibody epitopes used in each ELISA setting and/or possible heterogeneity in the molecular forms of sGPC3. Meta-analysis of the literatures has suggested that sGPC3 is indeed higher in HCC patients than normal subjects. However, its diagnostic utility is questionable, and further studies are required[55]. Moreover, prognostic analyses were not performed in those studies.

Very recently, we reported a novel sandwich ELISA system that recognized the N-terminal subunit of sGPC3 (sGPC3N)[56]. This ELISA system was highly sensitive compared to others that have been reported (Table 1). Using this ELISA system, sGPC3N antigen levels of 25 healthy volunteers and 115 HCC patients who had undergone curative partial hepatectomy were measured, and the relationship between sGPC3N and clinicopathological parameters was analyzed[56]. The mean ± standard deviation (SD) of sGPC3 levels in healthy controls was 110.12 ± 37.70 pg/mL, with a median value of 115.95 pg/mL. In HCC patients, sGPC3N levels were significantly increased compared to healthy controls and showed mean and median values of 405.16 pg/mL and 236.19 pg/mL, respectively. About 60% of HCC cases showed abnormally high sGPC3N levels (*i.e.*, > mean GPC3 + 2 SD of healthy controls) in preoperative sera and the levels declined significantly after curative partial hepatectomy[56]. Notably, we observed that high preoperative sGPC3N levels were significantly associated with shorter OS and DFS after hepatectomy and also with larger GPC3 IHC-positive areas in the resected HCC tissues. More importantly, multivariate analysis revealed that elevated sGPC3N was an independent prognostic marker for poor OS or DFS[56].

FUNCTIONS OF GPC3 IN HCC PROGRESSION

An important remaining question is whether GPC3 has a direct role in the aggressive behavior of HCC cells, or whether the expression is simply an epiphenomenon of malignant progression. Some experimental evidence has suggested that it is a direct relationship, but interpretation is complicated by the roles of cellular GPC3 in HCC cell biology. Cell surface GPC3 forms a complex with Wnt *via* its HS side chains and stimulates Wnt/β-catenin signaling in HCC cells (Figure 2)[57]. Sulfatase 2 (SULF2), an enzyme that removes 6-O-sulfate groups from HS, is also overexpressed in HCC cells and releases Wnt from the GPC3/Wnt complex, which also upregulates Wnt signaling[58]. Cell surface GPC3 may also act as a storage site for heparin-binding growth factors, such as FGF, hepatocyte growth factor (HGF) and heparin-

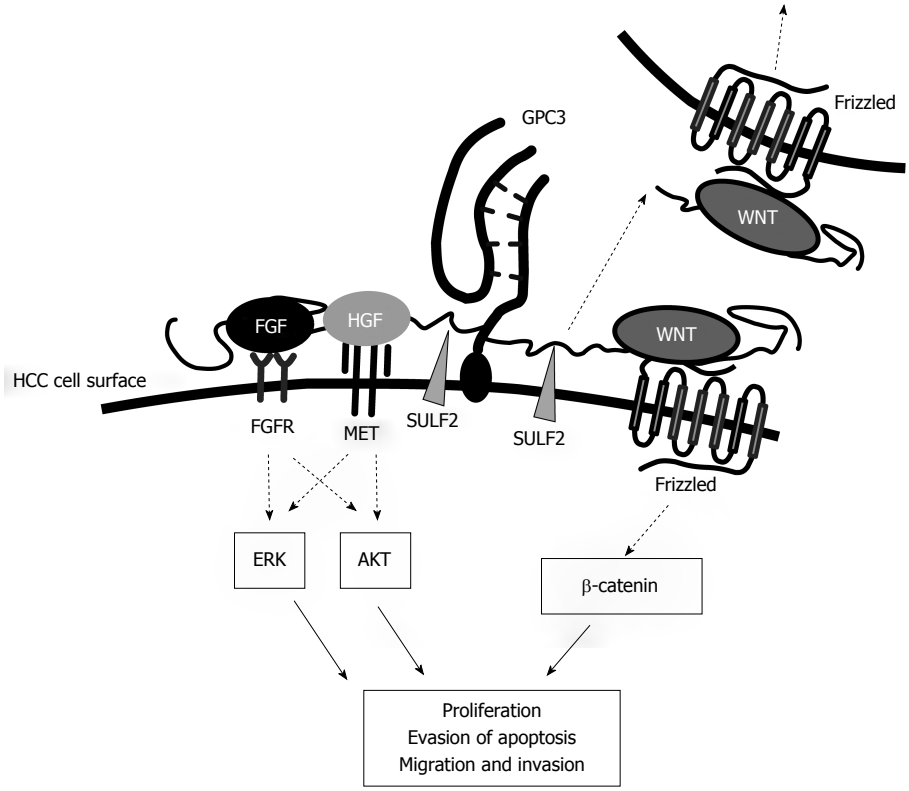

Figure 2 Possible roles of glypican-3 in progression of hepatocellular carcinoma. Cell surface glypican-3 (GPC3) forms a complex with Wnt and heparin-binding growth factors such as fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) *via* its heparan sulfate (HS) side chains and stimulates receptor-mediated signaling in hepatocellular carcinoma (HCC) cells. SULF2 expressed in HCC cells cleaves the HS to release these ligands from the GPC3/ligand complex, which may also upregulate signaling in neighboring HCC cells.

binding epidermal growth factor, all of which are likely involved in the invasive growth of HCC cells *via* ERK and/or AKT signaling (Figure 2)[59,60]. A recent study suggested that high expression levels of GPC3 may promote the epithelial-mesenchymal transition (EMT) of HCC cells through ERK activation[61], and EMT is known to be involved in the metastatic phenotype and drug resistance of cancer cells[62]. On the other hand, enhanced shedding of GPC3 with HS side chains may eliminate GPC3-attached Wnt and growth factors from HCC's cell surface or may show dominant-negative effects by neutralizing these GPC3-binding factors in the pericellular microenvironment[59]. Therefore, GPC3 may have both positive and negative effects on Wnt signaling and pericellular growth factor activities.

The oncoprotein c-Myc may also contribute to the presumed GPC3-induced malignant phenotypes. In the *GPC3* gene promoter region, Li *et al*[63] identified a binding site for c-Myc, and the binding of c-Myc directly activated the transcription of the *GPC3* gene. Interestingly, GPC3 also upregulated c-Myc expression, which eventually forms a positive feedback signaling loop between GPC3 and c-Myc in HCC cells[63].

Studies with GPC3-deficient mice indicated that loss of GPC3 function impaired the differentiation of macrophage lineage cells, resulting in the reduction of monocyte/macrophage precursor-derived osteoclasts in the diaphysis of the bone[64]. This evidence may suggest a relationship between macrophage function and GPC3. In many solid tumors, intratumoral infiltration of macrophages and the shift of their polarity to M2 phenotype have important roles in malignant progression[65]. Thus, we used IHC to assess the association of GPC3 expression with the number of tumor-associated macrophages (TAM) and their polarity in HCC tissues. Our results indicated that enhanced circumferential membranous staining of GPC3 (*i.e.*, a high A-Cm score) was associated with increased TAM with an M2-polarized phenotype[66,67]. We also observed a correlation between a high A-Cm score and the expression of monocarboxylate transporter 4 (MCT4) in HCC cells, and patients with MCT4-positive HCC also had poor prognoses[67]. MCT4 is an important proton symporter that regulates intracellular pH, and its expression is regulated by HIF1 signaling. Enhanced expression of MCT4 has been reported in many solid cancers[68]. It is currently unknown whether GPC3 expression and MCT4 expression are functionally related in HCC. Nonetheless, the pericellular microenvironment of HCC cells may have important roles in GPC3 expression.

GPC3-TARGETING IMMUNOTHERAPY

HCC is an extremely malignant tumor. Many researchers and clinicians are searching for novel

treatment strategies for this deadly disease, including molecular targeting therapy, immune therapy, oncolytic virotherapy and microRNA-based therapy[69-73]. The humanized anti-GPC3 monoclonal antibody GC33 shows significant cytotoxic activity against GPC3-expressing human HCC cell lines *in vivo* through ADCC and/or CDC[33,34]. The first clinical phase I study of humanized GC33 was performed in the United States, and the results were reported in 2013[74]. Twenty patients with advanced HCC were treated with humanized GC33 antibody (2.5-20 mg/kg, iv, weekly), and there were no dose-limiting toxicities[74]. Another phase Ⅰ trial in Japanese HCC patients also showed that GC33 given up to 20 mg/kg weekly was well tolerated[75]. A randomized phase II trial of humanized GC33 was performed in 185 advanced HCC patients, the results of which were presented at the 2014 ASCO meeting by Yen *et al*[76] Prior to randomization of the patients, they were separated into 3 groups based on the GPC3 expression levels judged by IHC. The 121 randomized patients were then treated with humanized GC33 (1600 mg every two weeks, iv, after two weekly doses), and 64 patients were treated with a placebo. Median progression-free survival (PFS) in the humanized GC33 and placebo groups were 2.6 and 1.5 mo, respectively (HR: 0.97, P = 0.87)[76]. Therefore, treatment of humanized GC33 did not show a benefit in this trial. However, exposure-efficacy analysis suggested that higher exposure of GC33 with FcγR3A-158V polymorphism or CD16 expression intensity may correlate with prolonged PFS[76]. As GC33 induces cytotoxic effects through ADCC and/or CDC, antibody concentration and efficacy of immune responses in HCC tissue might be critical. Further studies analyzing the immuno-microenvironmental factors in GPC3-expressing HCC are warranted.

GPC3 is also considered an immunotherapeutic target for peptide vaccine therapies[77-79]. Komori *et al*[78] developed HLA-A2 and -A24-restricted GPC3-derived peptide vaccines ($GPC3^{144-152}$: FVGEFFTDV and $GPC3^{298-306}$: EYILSLEEL, respectively). The patients who were induced by the peptides showed increased GPC3-specific cytotoxic T cells (CTLs). Then, by using these two peptides, a phase I trial was performed, in which 39 Japanese patients with advanced HCC were enrolled[80]. No severe common adverse events were observed, and one patient showed partial response and 19 patients showed stable disease. Notably, GPC3-specific CTLs were increased in 30 patients, and the frequency of the GPC3-specific CTLs correlated with OS[80]. On the other hand, development of T cells expressing GPC3-targeting chimeric antigen receptor was reported, which potently eliminated GPC3-positive HCC cell xenografts[81].

The chimeric proteins composed of an antibody fragment fused to a toxin (*i.e.*, immunotoxins) may also have a therapeutic potential in GPC3-targeting therapies. Recently, Gao *et al*[82] reported successful regression of tumor xenografts of two human liver cancer cell lines, Hep3B and HepG2, by treatment with anti-GPC3 immunotoxin. They used two anti-GPC3 monoclonal antibodies (HN3 and YP7) conjugated to *Pseudomonas* exotoxin A. HN3 inhibited Wnt signaling induced by GPC3[83] and YP7 recognized an epitope in the C-terminus portion of GPC3. HN3-immunotoxin treatment showed superior anti-tumor effects compared to YP7-immunotoxin[81].

CONCLUSION

GPC3 is frequently overexpressed in HCC, and its expression level serves as a promising prognostic biomarker. GPC3 may also be a promising molecular target for the development of innovative therapies to improve prognosis of HCC patients. Although a clinical benefit of GPC3-targeting therapy has not yet been confirmed in HCC patients, researchers are actively investigating novel strategies to develop GPC3-targeted therapies for the treatment of HCC.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Differentiation of hepatocellular carcinoma from its various mimickers in liver magnetic resonance imaging: What are the tips when using hepatocyte-specific agents?

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Abstract

Hepatocellular carcinoma is the most common primary hepatic malignant tumor. With widespread use of liver imaging, various cirrhosis-related nodules are frequently detected in patients with chronic liver disease, while diverse hypervascular hepatic lesions are incidentally detected but undiagnosed on dynamic computed tomography and magnetic resonance imaging (MRI). However, use of hepatocyte-specific MR contrast agents with combined perfusion and hepatocyte-selective properties have improved diagnostic performance in detection and characterization of focal liver lesions. Meanwhile, the enhancement patterns observed during dynamic phases using hepatocyte-specific agents may be different from those observed during MRI using conventional extracellular fluid agents, leading to confusion in diagnosis. Therefore, we discuss useful tips for the differentiation of hepatocellular carcinoma from similar lesions in patients with and without chronic liver disease using liver MRI with hepatocyte-specific agents.

Key words: Hepatocellular carcinoma; Gadoxetic acid; Magnetic resonance imaging; Liver cirrhosis

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Core tip: Hepatocellular carcinoma is the most common primary hepatic malignant tumor. With widespread use of liver imaging, various cirrhosis-related nodules are more frequently detected in patients with chronic liver disease, while diverse hypervascular hepatic lesions are incidentally detected but undiagnosed on dynamic computed tomography and magnetic resonance imaging (MRI). However, liver MRI using hepatocyte-specific agents has been suggested to be

a much more reliable modality in the detection and characterization of focal liver lesions. Therefore, we would like to discuss useful tips for the differentiation of hepatocellular carcinoma from similar lesions in patients with and without chronic liver disease using liver MRI with hepatocyte-specific agents.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignant neoplasm and the third most common cause of disease-related mortality worldwide[1,2]. Its incidence has been increasing in several countries. Meanwhile, in recent years, diagnostic imaging modalities for HCC have markedly improved, with technical advancements in multidetector row computed tomography (MDCT) and magnetic resonance imaging (MRI). Therefore, HCC is commonly diagnosed using dynamic CT and/or dynamic MRI without histological confirmation, on the basis of a characteristic arterial enhancement and portal venous or delayed phase washout[3,4]. For the radiological diagnosis of HCC, MRI is more sensitive (81% *vs* 68%) than CT, while their specificities are comparable (85% *vs* 95%)[5]. Moreover, liver MRI using hepatocyte-specific agents has been suggested to be much more reliable than other modalities such as MDCT and extracellular contrast-enhanced dynamic MRI[6].

Hepatocyte-specific MR contrast agents initially distribute in the extracellular fluid (ECF) compartment, similar to ECF contrast agents, and are subsequently taken up by functioning hepatocytes and excreted in the bile. Consequently, these agents provide dual benefits of dynamic imaging as well as delayed hepatobiliary phase (HBP) imaging[7]. Among the commercially available hepatocyte-specific agents, gadoxetic acid (Primovist outside the United States or Eovist in the United States, Bayer Healthcare, Berlin, Germany; formerly known as Gd-EOB-DTPA) is currently used widely because of the rapid acquisition of HBP images (10-20 min after contrast injection) and more intense HBP enhancement[7]. Furthermore, whereas dynamic MRI using hepatocyte-specific agents showed a performance comparable with that of CT with regard to focal lesion characterization, HBP significantly improved the diagnostic accuracy in terms of lesion detection and characterization[8-11].

On the other hand, with technical improvements in and the widespread use of liver MRI, various cirrhosis-related nodules are more frequently detected in patients with chronic liver disease, while diverse hypervascular hepatic lesions are incidentally detected but undiagnosed on CT. Consequentially, these hepatic lesions are mostly referred for MRI, particularly liver MRI using hepatocyte-specific agents, for lesion characterization. These diverse hepatic lesions may occasionally mimic HCC, resulting in a diagnostic challenge in clinical practice. Therefore, the aim of this study is to briefly describe the imaging findings of HCC and lesions mimicking HCC and discuss useful tips for the differentiation of HCC from similar lesions using liver MRI with hepatocyte-specific agents.

MRI FINDINGS OF HCC

On gadoxetic acid-enhanced MRI, HCC typically shows intense arterial enhancement and delayed washout (Figure 1). On HBP images, it usually shows low signal intensity (SI) in strongly enhanced normal hepatic parenchyma because of the absence of functioning hepatocytes or decreased expression of organic anionic transporting polypeptide (OATP), which is responsible for the intracellular uptake of contrast material[12]. On the other hand, 10%-20% of HCCs appear hypovascular during the hepatic arterial phase. Approximately 10% show high SI on HBP images because of OATP overexpression[12-14].

In addition, there are several ancillary features favoring the diagnosis of HCC[15]. Approximately 70% of lesions exhibit a tumor capsule or pseudocapsule[16] (Figure 2), which appears as a delayed enhancing rim, and the appearance of the capsule has been shown to be an important predictor of HCC[17]. A nodule-in-nodule appearance, suggesting the emergence of a progressed HCC within a dysplastic nodule (DN) or early HCC, is not frequently observed, although it is characteristic of HCC[18]. A mosaic appearance attributed to intratumoral heterogeneity is more common with larger HCCs, but not with tumors other than HCC[15].

On nonenhanced T1- and T2-weighted images, HCC shows variable SIs depending on the presence of iron, fat, or hemorrhage, although typically, low SI is observed on T1-weighted images and mild to moderately high SI is observed on T2-weighted images. HCC also typically demonstrates restricted diffusion. The findings of high SI on T2-weighted images and restricted diffusion are not specific to HCC, although they favor the diagnosis of malignancy and aid in the differentiation of HCC from cirrhotic nodules[15].

DIFFERENTIAL DIAGNOSIS

In patients with chronic liver disease

Regenerative and dysplastic nodules: Cirrhosis is characterized by the progressive fibrosis of the liver parenchyma and a spectrum of hepatocellular nodules

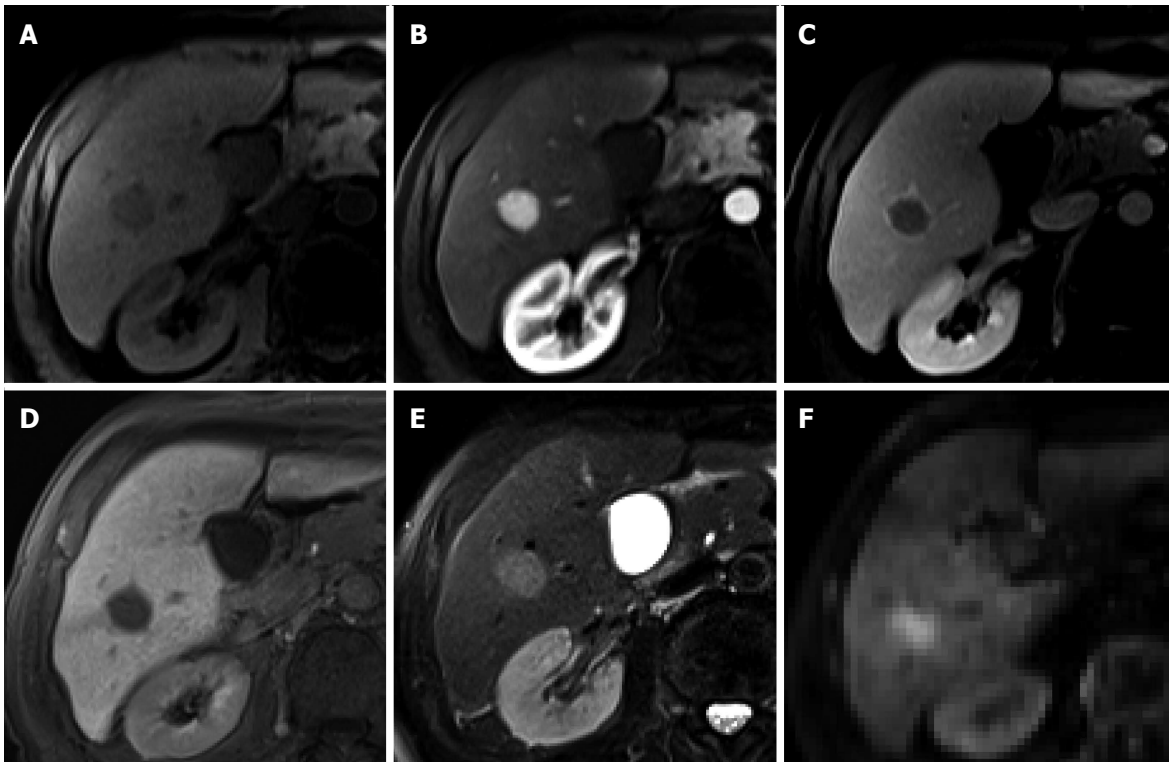

Figure 1 Hepatocellular carcinoma in a 74-year-old man with hepatitis C infection. A: Precontrast T1-weighted image shows a hypointense nodule in segment 6; B: Hepatic arterial phase of gadoxetic acid-enhanced MRI shows homogeneous marked enhancement of the tumor; C: Transitional phase shows washout of the contrast medium in the tumor with capsular enhancement; D: Hepatobiliary phase shows marked hypointensity of the tumor relative to the liver parenchyma; E, F: T2-weighted image and diffusion weighted image (*b* = 800) show high SI of the tumor.

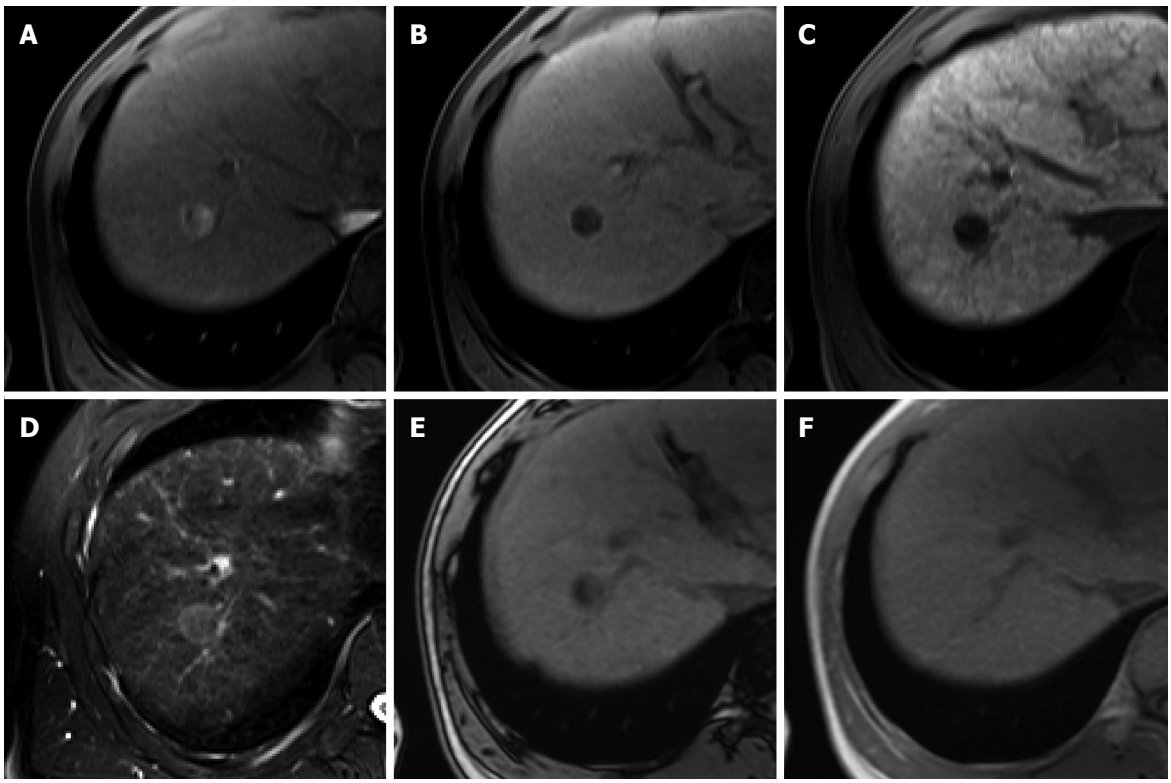

Figure 2 Fat-containing hepatocellular carcinoma in a 57-year-old man with hepatitis B infection. A: Hepatic arterial phase using gadoxetic acid shows heterogeneously arterial enhancement with intralesional low SI area; B: Transitional phase shows washout of the contrast medium in the tumor with capsular enhancement; C: Hepatobiliary phase shows marked hypointensity of the tumor relative to the liver parenchyma; D: T2-weighted image shows high signal intensity of the tumor; E, F: Opposed phase (E) and in-phase (F) T1-weighted gradient echo images reveals area of signal drop on opposed-phase image, indicating fat-containing lesion.

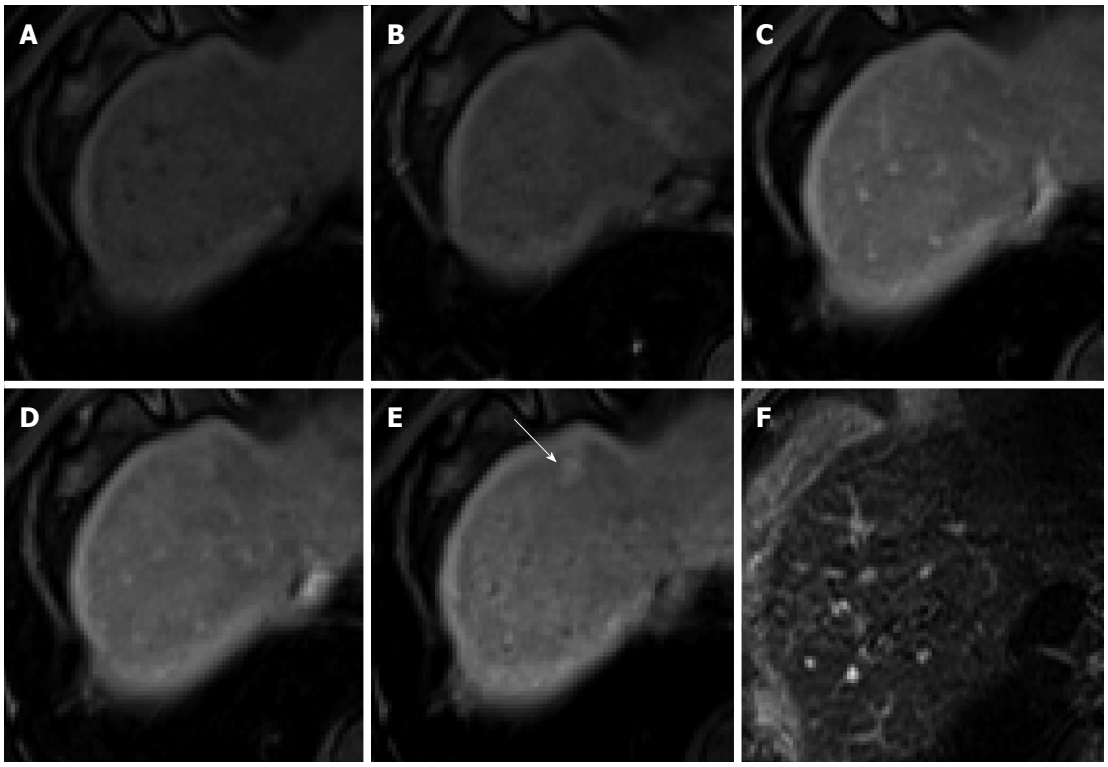

Figure 3 Regenerative nodule in a 61-year-old man with alcoholic liver cirrhosis. A-D: Precontrast T1-weighted image, hepatic arterial phase, portal venous phase, and transitional phase show no visible lesion in the scanned area; E: Hepatobiliary phase shows a hyperintense nodule (arrow) in hepatic S4; F: T2-weighted image shows isointensity of the tumor.

that mark the progression from regenerative nodules (RNs) to low- and high-grade dysplastic nodules (DNs) to, eventually, HCC[19,20]. There is a considerable overlap among these nodules during hepatocarcinogenesis on histopathology and imaging, although the characteristic imaging findings of hepatocarcinogenesis have been relatively well established[15,20].

RNs usually exhibit *iso* SI or low SI on T2-weighted images and variable SIs on T1-weighted images, while DNs characteristically exhibit high SI on T1-weighted images and *iso* SI or low SI on T2-weighted images. This is because DNs may contain more copper or iron compared with the background liver. Because these nodules may contain varying amounts of lipids, copper, or iron, they exhibit variable SIs on T1- and T2-weighted images depending on their content. However, RNs and DNs mostly do not exhibit high SI on T2-weighted images or restricted diffusion[21]. Therefore, during the differential diagnosis of RNs or DNs from HCCs, the presence of mild to moderately high SI on T2-weighted images or restricted diffusion strongly indicates the presence of HCC.

Following the injection of gadoxetic acid, because RNs are predominantly supplied by the portal vein, most of them enhance to the same degree as the adjacent liver parenchyma, resulting in *iso* SI in the hepatic arterial and later phases. Occasionally, they demonstrate slightly lower enhancement, which is observed as mildly low SI in the portal and transitional phases. Meanwhile, because DNs exhibit a decreased number of portal tracts with a relatively lesser increase in the number of unpaired arteries during hepatocarcinogenesis, they mostly demonstrate *iso* SI or low SI in the hepatic arterial and later phases. However, some DNs may have an increased arterial supply because of neoangiogenesis and enhance more than the liver in the hepatic arterial phase; this may lead to a misdiagnosis of hypervascular HCC[20].

On HBP images obtained using gadoxetic acid, RNs typically show *iso* SI or high SI because of preserved OATP expression[20] (Figure 3). DNs usually show *iso* SI or low SI on HBP images because of decreased OATP expression (Figure 4). Therefore, because OATP expression decreases during hepatocarcinogenesis, *iso* SI to high SI on HBP images is generally suggestive of benign lesions (RNs or low-grade DNs), while low SI on HBP images is a strong predictor of premalignancy or malignancy (high-grade DNs or HCCs).

Focal nodular hyperplasia-like nodules: Focal nodular hyperplasia (FNH)-like nodules are histopathologically and immunohistochemically identical to classic FNH observed in noncirrhotic livers, although they occur in patients with chronic liver disease or cirrhotic livers[22]. Therefore, the imaging findings of FNH-like nodules are also identical to the characteristic radiological findings of classic FNH on dynamic CT and MRI. Usually, FNH-like nodules are small hypervascular lesions[23] (Figure 5). However, if hypervascular FNH-like nodules are detected in cirrhotic livers, it is often difficult to differentiate them

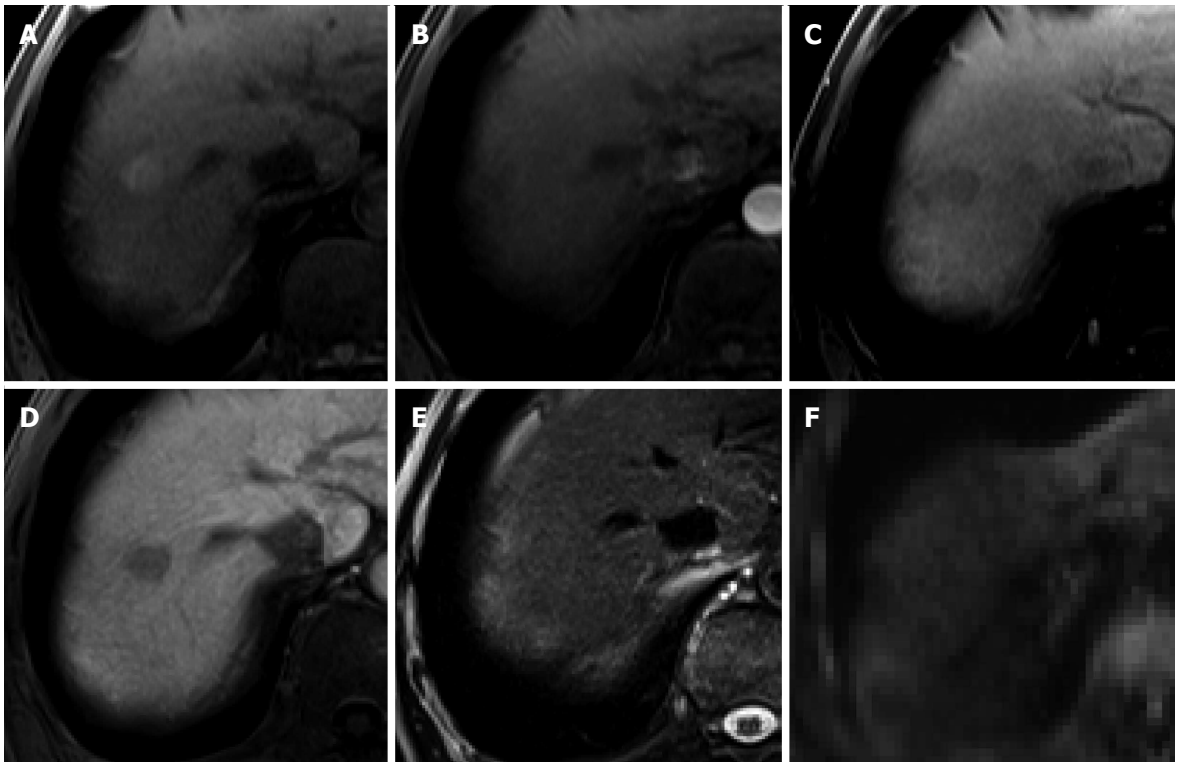

Figure 4 Dysplastic nodule in the same patient as Figure 1. A: precontrast T1-weighted image shows a hyperintense nodule in segment 8, suggesting high contents of iron or copper; B: Hepatic arterial phase of gadoxetic acid-enhanced MRI shows isointensity of the tumor; C, D: transitional and hepatobiliary phases show hypointensity of the tumor relative to the liver parenchyma; E, F: T2-weighted image and diffusion weighted image (b = 800) show isointensity of the tumor.

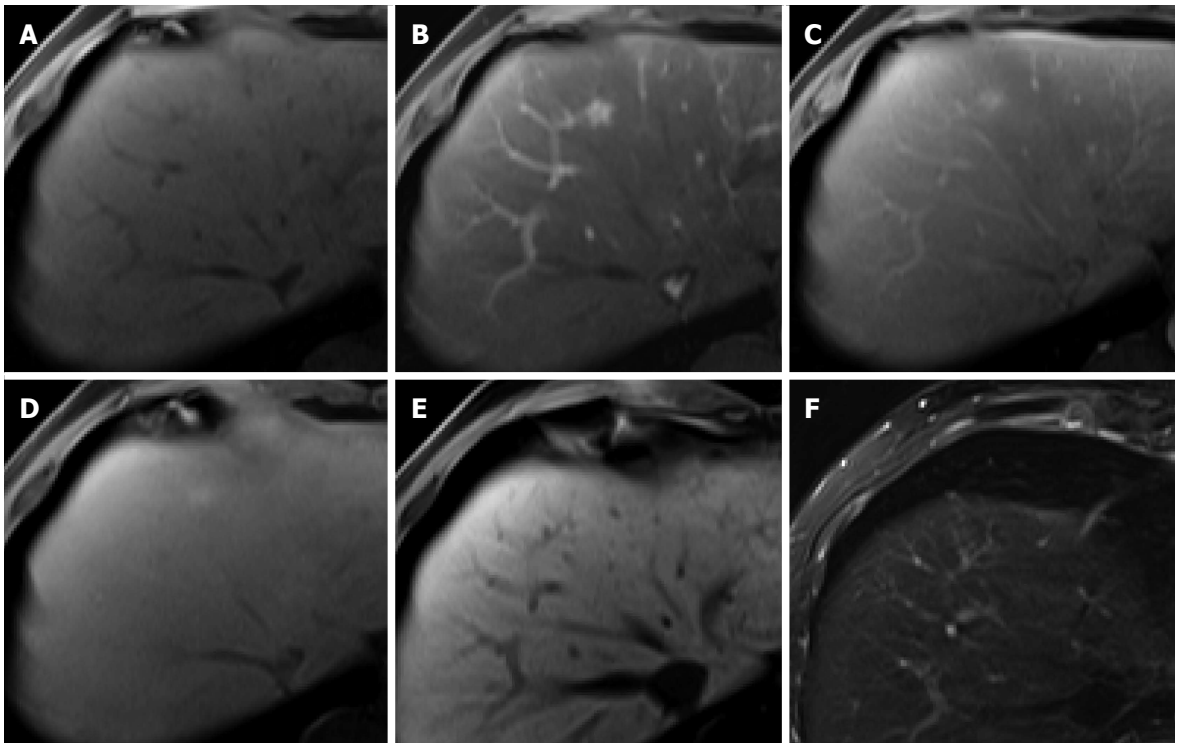

Figure 5 Focal nodular hyperplasia-like nodule in a 45-year-old man with hepatitis B infection. A: Precontrast T1-weighted image shows isointensity of the tumor; B: Hepatic arterial phase using gadoxetic acid shows lobulating-contoured, marked enhanced nodule in segment 4; C, D: Portal venous and transitional phases show slight hyperenhancement of the tumor relative to the liver parenchyma; E: Hepatobiliary phase shows isointensity or subtle peripheral ring-like enhancement of the tumor; F: T2-weighted image shows isointensity of the tumor.

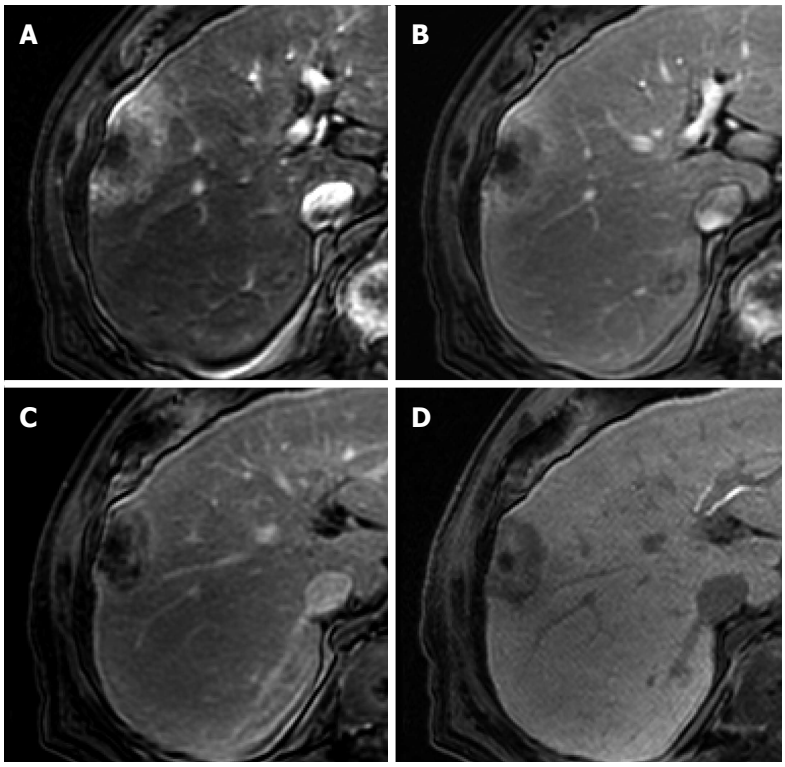

Figure 6 Intrahepatic cholangiocarcinoma in an 80-year-old man. A: Hepatic arterial phase using gadoxetic acid shows peripheral arterial enhancing mass with capsular retraction at the hepatic segment 8 subcapsular location; B: Portal venous and transitional phases show target appearance with peripheral enhancement and central nonenhancement; C, D: Hepatobiliary phase also shows low signal intensity of the most outer portion, high signal intensity of mid portion, and marked low signal intensity of the center of the tumor.

from HCC, particularly in atypical cases[24]. Because of identical imaging features, differences between atypical FNH-like nodules and typical HCCs will be discussed later in the FNH section. On the other hand, with regard to differentiation of typical FNH-like nodules from atypical HCCs with HBP high SI, lack of delayed washout is a key imaging finding for diagnosis of NRH (Kim JW, unpublished data, 2014)[25].

Nodular regenerative hyperplasia: Nodular regenerative hyperplasia (NRH) is a rare liver condition characterized by the widespread benign transformation of the hepatic parenchyma into small RNs. NRH may lead to the development of noncirrhotic portal hypertension[26] and is often associated with organ transplantation, myeloproliferative disease, or autoimmune processes. NRH exhibits *iso* SI to high SI on T1-weighted images (93.9%) and *iso* SI on T2-weighted images (82%), which are slightly different from the T1 and T2 SIs for FNH[27]. In one study using hepatocyte-specific MR agents with gadobenate dimeglumine[27], all NRHs showed arterial enhancement and *iso* SI to high SI on portal venous, equilibrium, and HBP images. Although the dynamic enhancement pattern of NRH resembles that of FNH, FNHs and FNH-like nodules show strong arterial enhancement and NRHs show mild arterial enhancement[27,28]. According to unpublished data of Kozaka *et al*[28], NRH appears as peripheral ring-like enhancement of the lesion on HBP images using gadoxetic acid, and they described this appearance as a doughnut-like nodule in HBP images. Therefore, arterial enhancement degree and SIs on T1- and T2-weighted images can provide differentiation of NRHs from FNHs and FNH-like nodules. Furthermore, the absence of washout and either *iso* to high SI or doughnut-like nodules on HBP images will distinguish this benign lesion from HCC.

Intrahepatic cholangiocarcinoma: Intrahepatic mass-forming cholangiocarcinoma (ICC) is the second most common primary hepatic malignancy after HCC. The typical enhancement pattern of ICC (77%) is peripheral rim-like arterial enhancement with progressive and concentric fill-in enhancement[29-31] (Figure 6). However, small ICC lesions (less than 3 cm in diameter, up to 6% of ICCs) can show an atypical enhancement pattern characterized by homogeneous arterial enhancement with washout, thus mimicking HCC[30] (Figure 6). Moreover, hepatitis C virus-induced liver cirrhosis has been recognized as an important risk factor for the development of ICC[32]. It may cause difficulty in the differential diagnosis of small hypervascular ICC from HCC in patients with liver cirrhosis, particularly that secondary to hepatitis C infection. Meanwhile, on gadoxetic acid-enhanced HBP images, most ICCs (96%) show low SI. In previous studies, 32%-85% of ICCs showed a central hyperintense area with a peripheral hypointense rim,

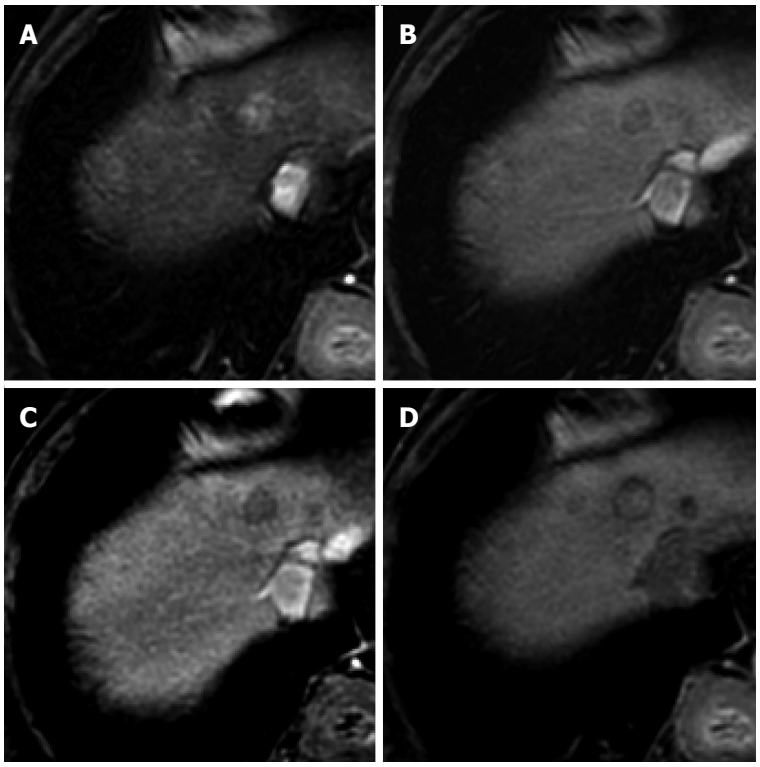

Figure 7 Intrahepatic cholangiocarcinoma in an 80-year-old man. A: Hepatic arterial phase using gadoxetic acid shows heterogeneously arterial enhancing nodule in hepatic segment 4 dome; B, C: Portal venous and transitional phases show delayed washout; D: Hepatobiliary phase shows target appearance with peripheral low signal intensity and central high signal intensity.

known as target appearance. Furthermore, a central high SI was described as an EOB cloud, attributed to contrast uptake by the central fibrotic stroma[30,31,33] (Figure 7). In a comparison between ICC and HCC using gadoxetic acid-enhanced MRI[33], the target appearance on HBP images was more common with ICC than with HCC (85.7% *vs* 17.1%) and was the best predictor for distinguishing ICC from HCC. Furthermore, HBP images were found to show an increased lesion conspicuity (lobulated shape of ICC *vs* globular shape of HCC) and better delineation of daughter nodules and intrahepatic metastasis[30], which may aid in ICC diagnosis.

In patients without chronic liver disease or with a normal liver

Focal nodular hyperplasia: FNH is the second most common benign hepatic tumor, found more commonly in healthy young and middle-aged women. Histologically, FNH is characterized by a central fibrous scar with surrounding nodules of hyperplastic hepatocytes and small bile ducts. Because of the benign nature of FNH, which usually necessitates conservative management, noninvasive diagnosis is important. Characteristic morphological features and dynamic enhancement patterns have been well demonstrated for FNH[34]. Morphologically, FNH shows a lobulated or microlobulated border without a true tumor capsule and has a central fibrous scar. Similar to the typical imaging findings observed on dynamic CT and MRI using conventional ECF agents, FNH shows intense and homogeneous arterial enhancement that subsequently fades without delayed washout and *iso* SI or high SI in the portal venous and transitional phases of gadoxetic acid-enhanced MRI[34,35]. Because of continuous contrast uptake by functioning hepatocytes within the tumor, the majority (91%-96%) of FNHs show *iso* SI or high SI on HBP images[34-37]. *Iso* SI or high SI on HBP images is a characteristic imaging finding of FNH, allowing accurate diagnosis[35,37-39]. Although a small number of FNHs (23%) show mixed or low SI on HBP images, peripheral ring-like enhancement of the lesion is frequently observed and is crucial for the identification of FNH[36] (Figure 8). On the other hand, the presence of a typical central scar is a reliable radiological sign for FNH diagnosis. However, a macroscopic central scar occurs in 50%-61% of FNHs and is often absent in FNHs measuring less than 3 cm[37,40]. Compared with that observed using ECF contrast agents, a central scar observed using gadoxetic acid does not typically demonstrate delayed enhancement, resulting in markedly low SI on HBP images[41]. Accordingly, FNH with a large central scar rarely shows low SI on HBP images. Because approximately 10% of HCC and less than 10% of FNH lesions show high and low SI, respectively, on HBP images[13,34,35,37], a definitive diagnosis of HCC and FNH is sometimes difficult. However, with regard to hypervascular FNH with low SI on HBP images, female sex, presence in the normal liver, characteristic morphologic features such as

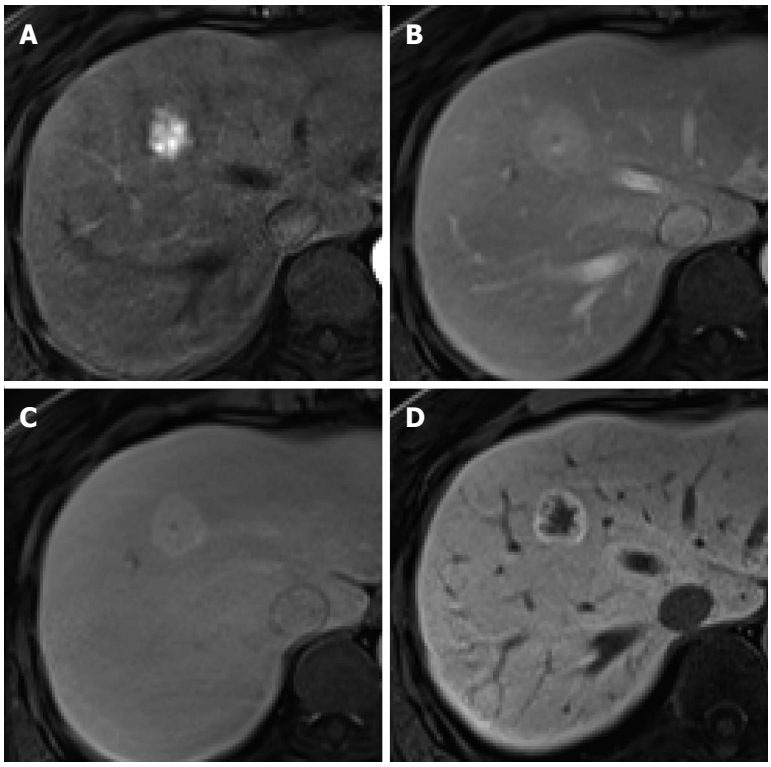

Figure 8 Focal nodular hyperplasia in a 55-year-old woman. A: Hepatic arterial phase image of gadoxetic acid-enhanced etic resonance imaging shows lobulating contoured, marked enhanced tumor; B, C: Portal venous and transitional phases show slightly hyperenhancement of the tumor relative to the liver parenchyma, and central hypointense area is suspected as central scar; D: Hepatobiliary phase shows peripheral ring-like enhancement of the tumor with larger area of markedly hypointense central scar as compared with the other phase images.

lobulated or microlobulated borders and a central scar, lack of delayed washout, and ring-like enhancement with central *iso* SI to low SI on HBP images are helpful for the diagnosis of FNH.

Hepatocellular adenoma: Hepatocellular adenoma (HCA) is the third most common benign hepatic tumor that particularly affects young and middle-aged women. It was recently subclassified into four groups according to the genotype and phenotype: inflammatory (50%), hepatocyte nuclear factor (NHF)-1α-mutated (35%-40%), β-catenin-mutated (10%-15%), and unclassified (< 10%)[42]. The MRI findings vary on the basis of histological findings and associated complications, and MRI has proven to be an accurate method for subtyping HCAs[43-45]. For several years, the differentiation of HCA from FNH has been a major concern, because these hypervascular tumors are frequently observed in women of a similar age. HCA requires surgical resection because of the risk of hemorrhage and malignant transformation[35,38,39]. On the other hand, on gadoxetic acid-enhanced MRI, 90% of HCAs show mild-to-moderate arterial enhancement, 72% show low SI in the transitional phase, and 93% show low SI on HBP images[35]. In particular, HNF-1α-mutated and β-catenin-mutated HCAs show arterial enhancement with washout and low SI on HBP images, mimicking HCC, while some inflammatory HCAs show arterial enhancement with persistent enhancement and high SI on HBP images, mimicking FNH[35,46]. Therefore, the majority of HCAs shows the typical enhancement pattern shown by HCC. However, the most important fact is that HCA typically occurs in noncirrhotic livers in women of child-bearing age, while HCC primarily occurs in cirrhotic livers. Notwithstanding, differentiation between HCA and HCC in noncirrhotic livers is an issue. However, a larger fat component is more typical for HNF-1α-mutated HCA[44]. A rim-like band with high SI in the periphery of the lesion on T2-weighted images (atoll sign) is observed for 13% of HCA[39,43]. Furthermore, a capsule or a pseudocapsule, which appears as a delayed enhancing rim, is observed less commonly in HCA than in HCC (Figure 9) (25%-31% *vs* 70%)[16,47,48]. Although there is no study on the advantages of using gadoxetic acid for the differential diagnosis of HCA and HCC, these ancillary findings will be helpful in their differentiation in noncirrhotic livers.

Hemangioma: Hemangioma is the most common benign hepatic tumor[49]. On dynamic CT and MRI using conventional ECF agents, a typical hemangioma shows early peripheral nodular enhancement with centripetal and prolonged enhancement (Figure 10). High-flow hemangiomas, which account for 16% of all hemangiomas and 42% of hemangiomas measuring less than 1 cm in diameter, show immediate homogeneous arterial enhancement with persistent enhancement in the portal and equilibrium phase[49]. With regard to

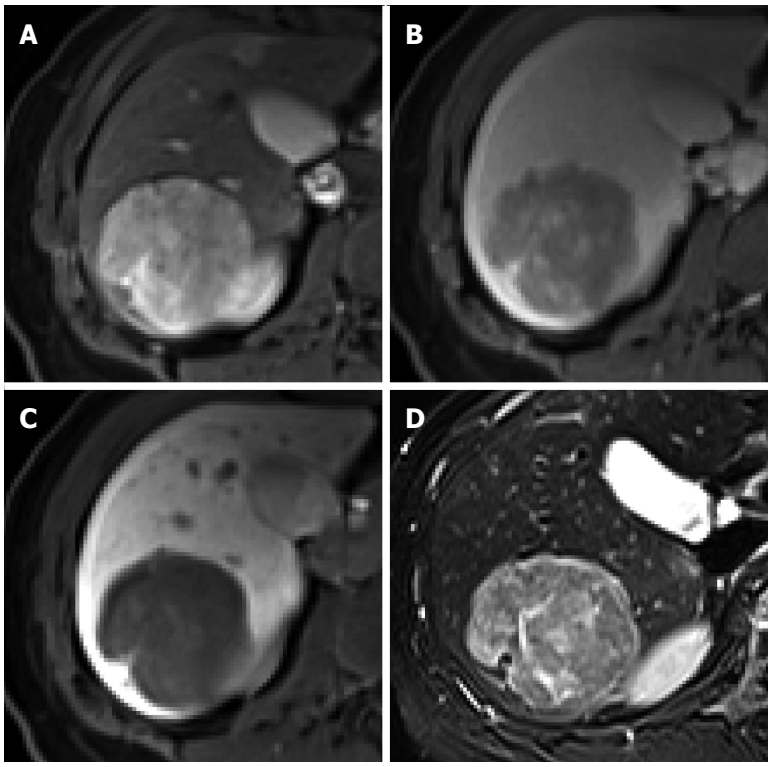

Figure 9 Hepatocellular adenoma in a 45-year-old woman. A: Hepatic arterial phase using gadoxetic acid shows moderate arterial enhancement; B: Transitional phase shows delayed washout without capsule or pseudocapsule; C: Hepatobiliary phase shows heterogeneous low signal intensity of the tumor; D: T2-weighted image shows a peripheral hyperintense band with moderate high signal intensity of residual tumor.

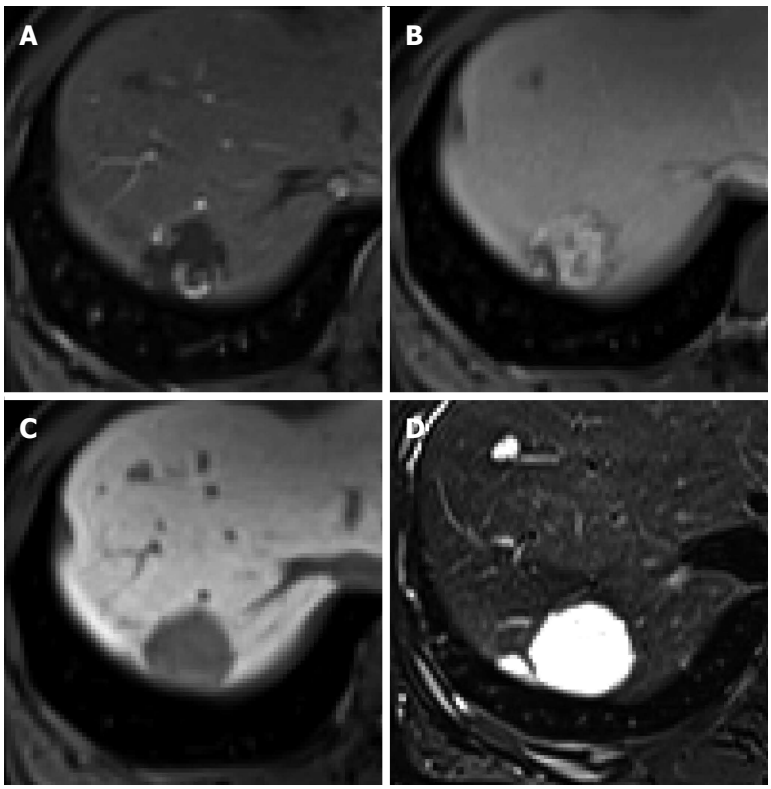

Figure 10 Hepatic hemangioma in a 45-year-old woman. A: Hepatic arterial phase using gadoxetic acid shows peripheral nodular enhancement of the tumor in segment 7; B: Transitional phase shows centripetal and prolonged enhancement; C: Hepatobiliary phase shows hypointense defect relative to hepatic parenchyma; D: T2-weighted image shows bright and high signal intensity.

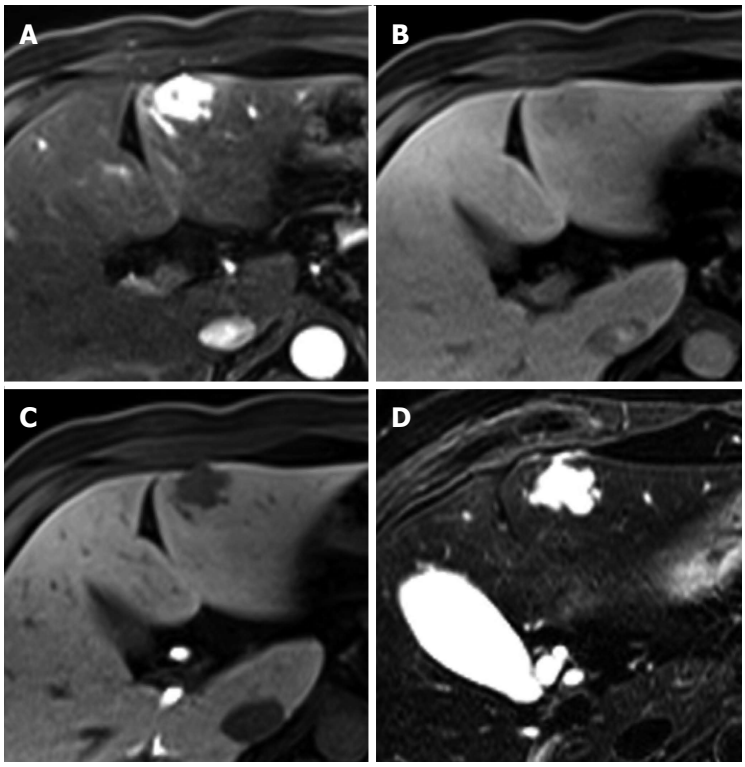

Figure 11 Hepatic hemangioma in a 54-year-old man. A: Hepatic arterial phase in gadoxetic acid-enhanced MRI shows homogeneous marked enhancement of the tumor in segment 3; B: Transitional phase shows slightly low signal intensity of the tumor relative to the liver parenchyma; C: Hepatobiliary phase shows more markedly low signal intensity of the tumor; D: T2-weighted image shows bright and high signal intensity of the tumor.

gadoxetic acid-enhanced MRI, hemangioma shows typically low SI on HBP images because of the absence of hepatocytes in the lesion[50]. Furthermore, it shows low SI in the transitional phase (Figure 11). Because contrast uptake by hepatocytes begins as early as the portal venous phase and parenchymal enhancement gradually increases in the transitional phase and HBP, hemangioma shows a relatively decreased SI compared with the parenchyma, exhibiting washout in the transitional phase[50]. Although this has been described as "pseudowashout"[51], hemangioma shows SI equivalent to that of the portal vein in all phases, with a gradual decrease in SI from the portal phase to HBP (no rapid washout)[50]. Moreover, hemangioma shows typically bright and high SI on T2-weighted images and high SI on diffusion-weighted images with high apparent diffusion coefficient value[51,52]. Therefore, high-flow hemangioma on gadoxetic acid-enhanced MRI, which shows arterial hyperenhancement with pseudowashout, can be confused for HCC. However, bright and high SI on T2-weighted images, high apparent diffusion coefficient value, and SI equivalent to that of the portal vein in all phases may be helpful for the differentiation of hemangioma from HCC.

Angiomyolipoma: Angiomyolipoma (AML) is a benign, nonencapsulated mesenchymal tumor comprising varying proportions of three tissue elements: blood vessels, smooth muscle, and mature adipose tissue. The presence of fat tissue is the most important radiological feature of AML, although it is not specific for AML. The fat component of AML varies from less than 10% to more than 90% of the tumor volume, resulting in a varied imaging appearance and leading to an erroneous diagnosis in most cases. In addition, the epithelioid type of AML, which contains no or a minimal amount of macroscopic fat, demonstrates arterial enhancement and delayed washout, mimicking HCC[53]. Therefore, AML has been commonly misdiagnosed as HCC. On dynamic CT and MRI using conventional ECF agents, the fatty areas of AML are well vascularized and show early enhancement, whereas steatotic foci in HCC are relatively avascular and show less contrast enhancement[54]. With regard to the vascular components of AML, tortuous central tumoral vessels and early draining veins were found to be pathognomonic features of AML[55]. On the other hand, only one study differentiated between AML and HCC using gadoxetic acid-enhanced MRI[56]. Kim *et al*[56] reported that 100% of AMLs and 85% of HCCs showed arterial enhancement and delayed washout on MRI using gadoxetic acid. Compared with HCC, AML was found to show homogeneous low SI on HBP images more frequently (83% *vs* 41%) (Figure 12), while the degree of enhancement for this lesion on HBP images was found to be much lower than that for the spleen (92% *vs* 30%). They explained that AML is devoid of hepatocytes, leading to a more homogeneously lower SI on HBP images, while HCC may contain some dysplastic hepatocytes, leading to a more

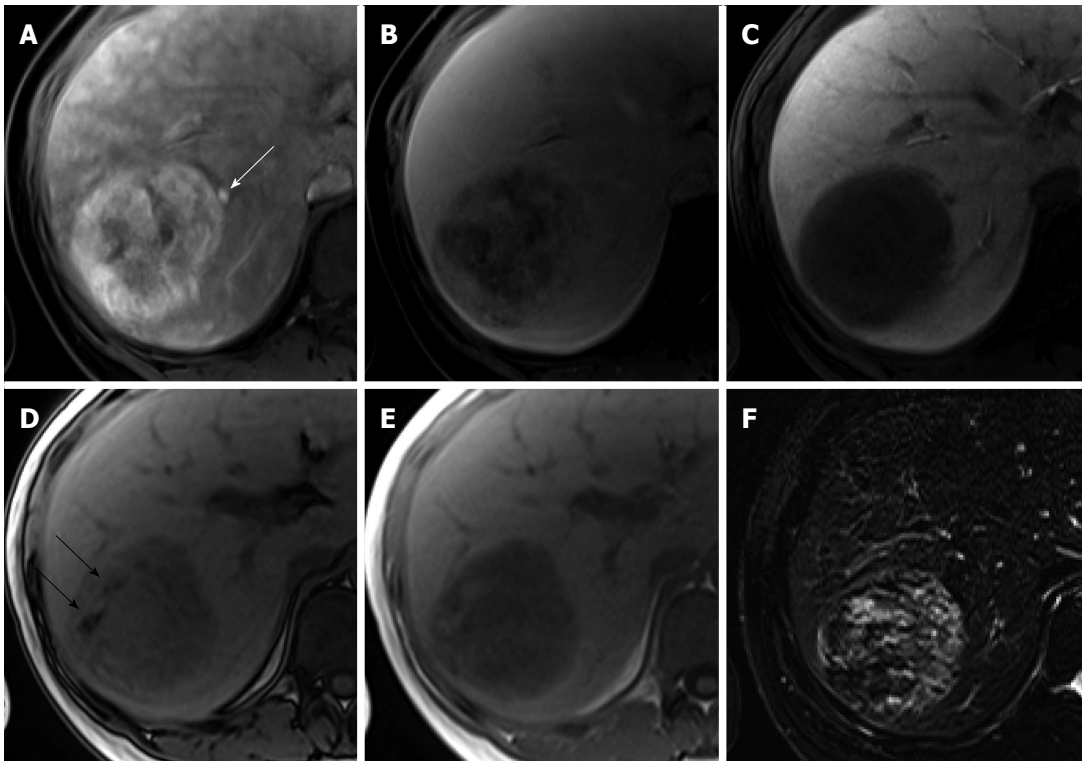

Figure 12 Angiomyolipoma in a 33-year-old woman. A: Hepatic arterial phase using gadoxetic acid shows heterogeneous arterial enhancement of the tumor. Early venous drainage to the right hepatic vein is seen (white arrow); B: Transitional phase shows heterogeneously low signal intensity; C: Hepatobiliary phase shows homogeneously low signal intensity; D, E: Opposed phase (D) and in-phase (E) T1-weighted gradient echo images reveal hypointense mass with area of signal drop on opposed-phase image (black arrows), indicating fat-containing lesion; F: T2-weighted image shows heterogeneous signal intensity of the tumor.

heterogeneously higher SI on HBP images. Therefore, HBP of gadoxetic acid-enhanced MRI would be the most beneficial sequence for discriminating AML from HCC. In addition, because AML is common in women, younger patients, and patients with a normal liver, the occurrence of lesions in young women with a normal liver would be a helpful clue for its differential diagnosis.

Focal eosinophilic infiltration: Focal eosinophilic infiltration (FEI) is a focal hepatic lesion caused by eosinophil-induced tissue damage. It is associated with various eosinophilia-related conditions such as parasitic infections, allergic reactions, hypereosinophilic syndrome, and internal malignancies. On imaging, FEI lesions appear as small, ill-defined, nonspherical lesions, with low attenuation in the portal phase during dynamic CT[57]. Nevertheless, radiological differentiation of FEI from hepatic metastasis is difficult because of its multiplicity and higher incidence in patients with an underlying malignancy[58,59]. Furthermore, arterial hypervascularity is infrequently observed in FEI[60]. On gadoxetic acid-enhanced MRI in particular, we observed that FEI showed arterial enhancement [70%; rim (37.1%) and homogeneous (22.9%) enhancement] and low SI in the portal venous phase, transitional phase, and HBP (80%, 88.6%, and 100%, respectively), resulting in 45.7% of lesions showing the typical enhancement pattern of HCC[61] (Figure 13). However, FEI was found to show characteristically mixed or intermingled low SI, irregular margins, and nonspherical shapes on HBP images, which would be helpful for characterizing this lesion[62]. Size discrepancy on HBP images relative to the size on T1- or T2-weighted images is another characteristic finding of FEI, although there are studies on the differentiation of FEI from metastasis using gadoxetic acid[58,59,62]. In addition, *iso* SI on T1-weighted images is a useful MRI finding for the diagnosis of FEI[59].

Hypervascular pseudolesions: Hypervascular pseudolesions, also known as arterioportal shunts, are typically wedge-shaped lesions with arterial enhancement in a subcapsular location. Typical lesions are easy to recognize and diagnose[63]. Meanwhile, subcentimeter-sized, small hypervascular pseudolesions tend to be nodular in shape[64] and are one of the primary lesions mimicking HCCs, resulting in difficulties in differential diagnosis. In HBP of gadoxetic acid-enhanced MRI, most hypervascular pseudolesions (94.3%) show *iso* SI compared with the surrounding liver tissue (Figure 14), which can be attributed to the intact hepatocyte function of these lesions[65], whereas up to 13% of nodular hypervascular pseudolesions show low SI on HBP images[64], also commonly demonstrated by HCCs. However, SI on HBP images is significantly lower for HCCs than for pseudolesions. Therefore, even though hypervascular pseudolesions rarely show low SI on HBP images, this finding may be

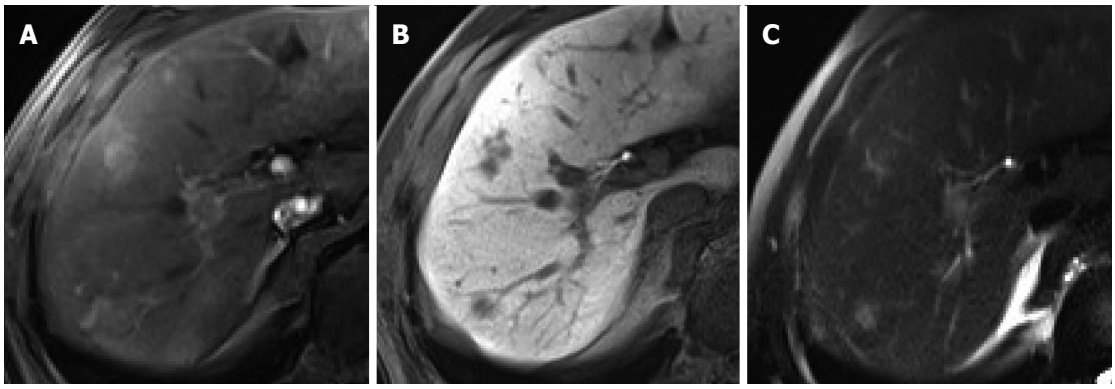

Figure 13 Focal eosinophilic infiltration in a 52-year-old man. A: Hepatic arterial phase of gadoxetic acid-enhanced MRI shows two irregular homogeneously enhancing nodular lesions in segments 7 and 8; B: Hepatobiliary phase shows low signal intensities with ill-defined margin and nonspherical shape; C: Heavily T2-weighted image shows smaller size of the lesions in S8, compared to (B).

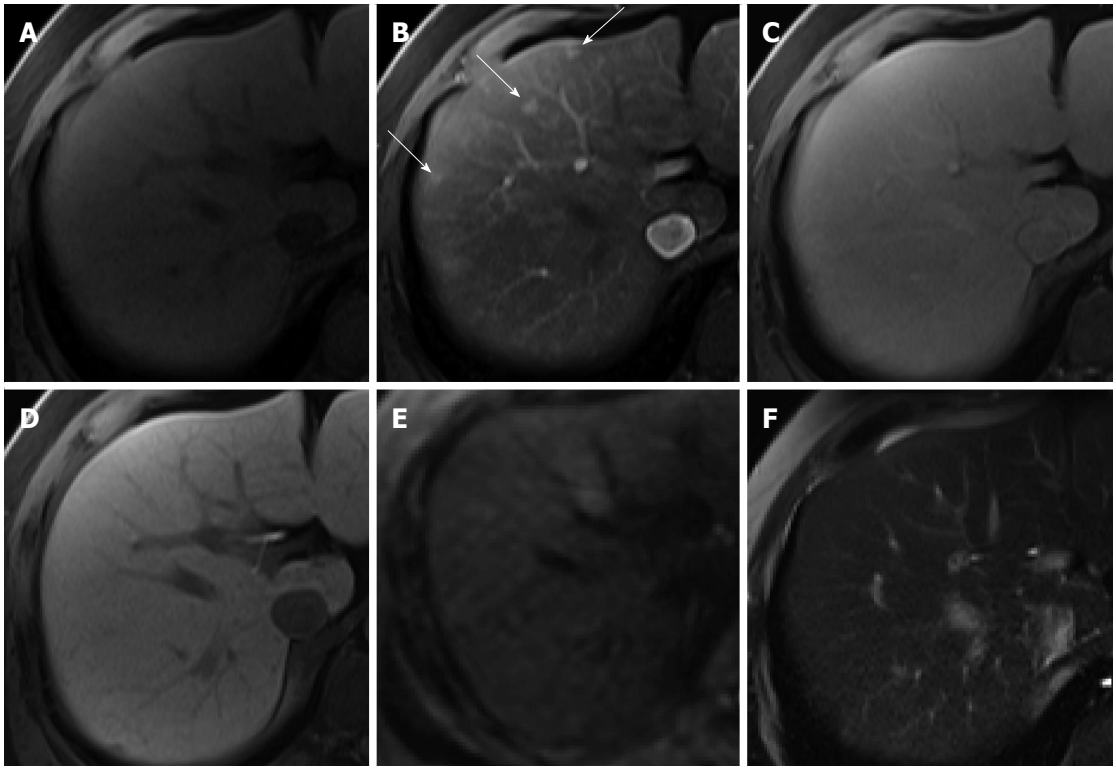

Figure 14 Hypervascular pseudolesion in a 49-year-old man with normal liver. A: Precontrast T1-weighted image shows no focal hepatic lesion; B: Hepatic arterial phase using gadoxetic acid shows several arterial enhancing nodular lesions in the liver (arrows); C-F: Transitional, hepatobiliary, T2-weighted, and diffusion weighted images show no signal change.

helpful for accurate differentiation from HCC.

Metastasis: Metastases are the most common malignant hepatic tumor. Metastases usually manifest as multiple discrete nodules or masses but occasionally manifest as a solitary nodule or mass[66]. Hepatic metastases from adenocarcinoma, such as colorectal cancer, are usually hypovascular and have arterial rim-like enhancement. Characteristically, neuroendocrine tumor, renal cell carcinoma, melanoma, and breast cancer are well known for hypervascular metastasis, showing arterial enhancement with delayed washout, like HCC. Although there have been few studies on enhancement pattern using gadoxetic acid, they have been commonly identified as defects on HBP images, owing to no functioning hepatocytes within the tumor[67]. However, in colorectal cancer liver metastasis, homogeneous defects on HBP were not common (27.8%) and heterogeneous defects on HBP were most common (63.3%)[68] (Figure 15). In breast cancer liver metastasis, this commonly manifested as a target sign (62%) with central high SI and peripheral low SI rim on the HBP, like ICC, because of contrast pooling at the central fibrotic area[67] (Figure 16). Therefore, hepatic metastases may appear as homogeneous defects, heterogeneous

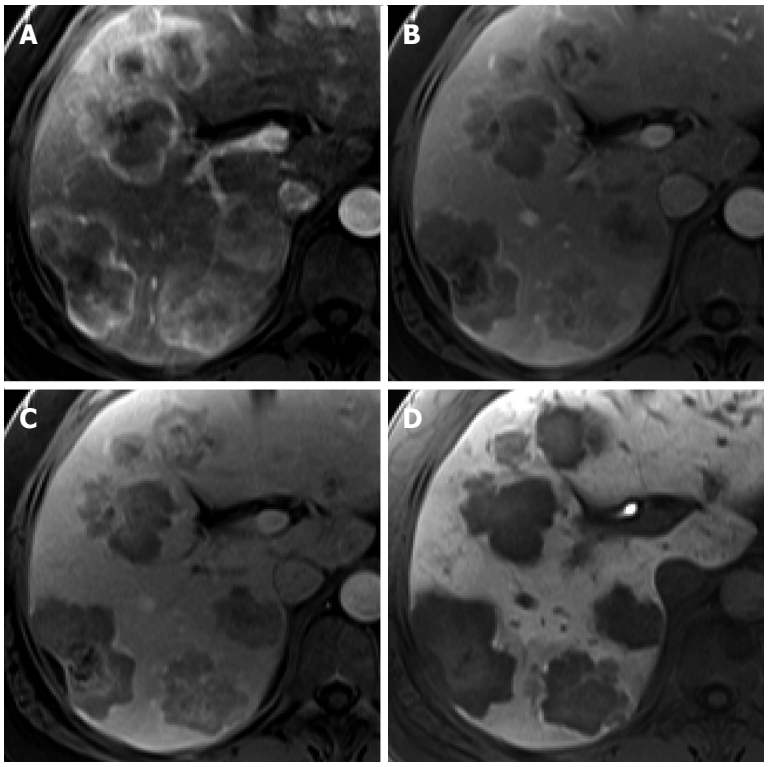

Figure 15 Hepatic metastasis from colon cancer in a 74-year-old man. A: Hepatic arterial phase using gadoxetic acid shows multiple arterial rim-like enhancing tumors with lobulating margin; B, C: Portal venous and transitional phases show heterogeneously low signal intensity of the tumors; D: Hepatobiliary phase shows peripheral hypointense rim with subtle high signal intensity of the center.

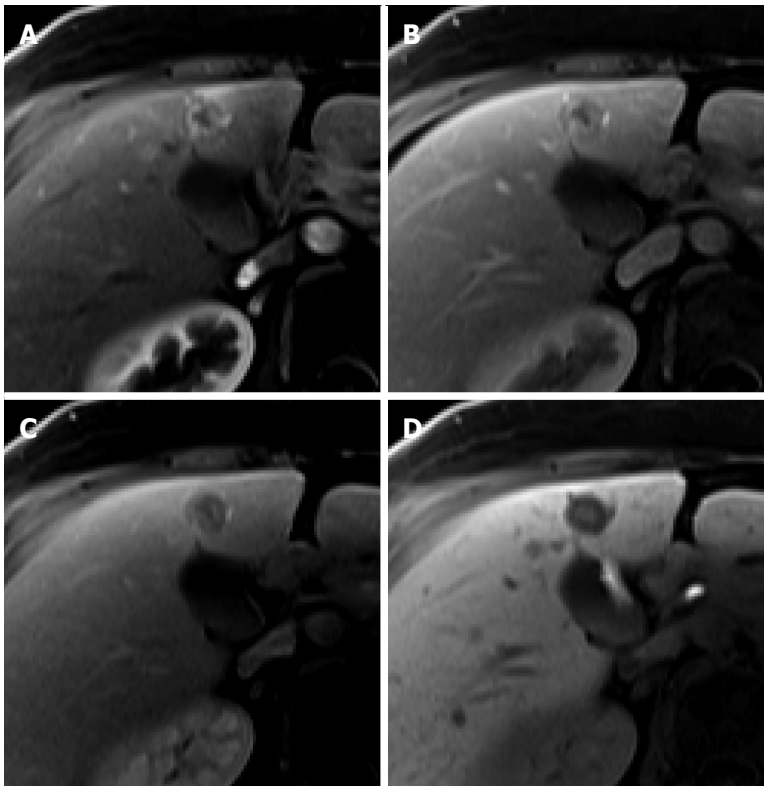

Figure 16 Hepatic metastasis from breast cancer in a 61-year-old woman. A: Hepatic arterial phase using gadoxetic acid shows arterial rim-like enhancement; B, C: Portal venous and transitional phases show delayed washout of periphery of the tumor with central nonenhancement; D: Hepatobiliary phase shows target appearance with subtle high signal intensity of the center and peripheral low signal intensity rim.

defects, or with target appearance on HBP images and the most common finding on HBP may vary from tumor to tumor. Meanwhile, the presence of multiple focal lesions in the non-cirrhotic liver of a patient with known malignancy, a homogeneous or heterogeneous defect, or target appearance on HBP image favor the diagnosis of metastasis.

CONCLUSION

In conclusion, we have described the key differentiating features for HCCs and lesions mimicking HCCs on gadoxetic acid-enhanced MRI for patients with and without chronic liver disease. HBP images obtained using gadoxetic acid provide useful information for the detection and characterization of focal hepatic lesions, although it should be noted that the appearance on HBP itself does not replace the information provided by the static and dynamic phases. However, the enhancement patterns observed during dynamic phases in gadoxetic acid-enhanced MRI may be different from those observed during MRI using conventional ECF agents, leading to confusion in diagnosis. Therefore, in addition to the contrast enhancement patterns on dynamic phase and HBP images, ancillary findings such as tumor appearance in each static phase and on other sequences such as T1- and T2-weighted images, along with clinical information, can aid in precise diagnosis.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Hepatocellular carcinoma mouse models: Hepatitis B virus-associated hepatocarcinogenesis and haploinsufficient tumor suppressor genes

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Abstract

The multifactorial and multistage pathogenesis of hepatocellular carcinoma (HCC) has fascinated a wide spectrum of scientists for decades. While a number of major risk factors have been identified, their mechanistic roles in hepatocarcinogenesis still need to be elucidated. Many tumor suppressor genes (TSGs) have been identified as being involved in HCC. These TSGs can be classified into two groups depending on the situation with respect to allelic mutation/loss in the tumors: the recessive TSGs with two required mutated alleles and the haploinsufficient TSGs with one required mutated allele. Hepatitis B virus (HBV) is one of the most important risk factors associated with HCC. Although mice cannot be infected with HBV due to the narrow host range of HBV and the lack of a proper receptor, one advantage of mouse models for HBV/HCC research is the numerous and powerful

genetic tools that help investigate the phenotypic effects of viral proteins and allow the dissection of the dose-dependent action of TSGs. Here, we mainly focus on the application of mouse models in relation to HBV-associated HCC and on TSGs that act either in a recessive or in a haploinsufficient manner. Discoveries obtained using mouse models will have a great impact on HCC translational medicine.

Key words: Hepatocellular carcinoma; Mouse models; Hepatitis B virus; Haploinsufficiency; Tumor suppressor genes

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Core tip: Hepatitis B virus (HBV) viral products, in particular the oncogenic HBV X protein, and mutations of tumor suppressor genes (TSGs) are the driving force of hepatocellular carcinoma (HCC). Inactivation of a recessive TSG requires mutations in both alleles and fits the "two-hit" model. However, haploinsufficiency occurs when one allele is insufficient to confer the full functionality of a TSG; the gene's effect can be partial or complete depending on tissue type, genetic modifiers/background, and environmental factors. Mouse models play a pivotal role in demonstrating the oncogenic effects of viral products and in establishing the dose-dependency and quantitative differences when analyzing a TSG involved in HCC.

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INTRODUCTION

Liver cancer is a member of the top ten most common cancers in both men and women[1]. The most common type of primary liver cancer in adults is hepatocellular carcinoma (HCC), which is derived from hepatocytes and accounts for 70% to 80% of cases[2,3]. Liver cancer has a high mortality and poor prognosis because patients usually are diagnosed with the disease at a well-advanced stage, making liver resection or transplantation unavailable as a therapeutic option. In addition, the recurrence rate of HCC is high in patients who have received liver resection surgery, resulting in a poor cure rate and low long-term survival[1,4]. At present, liver cancer is still a worldwide health issue and remains an unsolved medical problem.

The major risk factors for HCC include: (1) hepatitis virus infection, for example hepatitis B virus (HBV) and hepatitis C virus (HCV); (2) aflatoxin B1 (AFB1) exposure; (3) alcoholic cirrhosis; and (4) metabolic factors such as obesity and diabetes[5-8]. Furthermore, in HBV-associated HCC, HBV replication and the genotype of the HBV *per se* are also risk factors. A higher serum level of HBV DNA is correlated with the future incidence of HCC in patients[9]. In a manner similar to the HBV infection rate, the distribution of HBV genotypes also shows significant geographical differences. For example, genotypes B and C are predominant in Asia, while genotypes A and D are predominant in Africa, Europe and India. In addition, in Asia, genotype C has been shown to be correlated with advanced liver disease, cirrhosis and HCC more than genotype B[1]. Moreover, there is a highly significant association between patient gender, HBV infection and HCC. Males have a higher HBV infection rate and a higher HCC incidence. The greater susceptibility of males in terms of HCC incidence may be attributed to the tumor-promoting effects of androgens and/or the gender-specific metabolism of carcinogens[10]. AFB1, which is produced by fungi and often contaminates maize and peanuts, is a well-recognized carcinogen of the liver. A metabolic intermediate of AFB1 is able to bind to and damage DNA, leading in HCC to a loss of function of various tumor suppressor genes such as *p53*[10]. Notably, areas with a high prevalence of chronic HBV infection are usually places having a high risk of AFB1 exposure. This suggests that AFB1 is a promoting factor for HBV-associated HCC and that these two risk factors may have a synergistic effect on hepatocarcinogenesis. Another risk factor related to diet is chronic alcoholism, which leads to fatty liver, fibrosis, advanced liver disease and eventually to HCC. Additionally, smoking has been recognized as a risk factor for HBV-associated HCC development. Furthermore, genetic polymorphisms present in the host are also HCC risk factors. For example, studies have revealed that a loss-of-function deletion of glutathione S-transferase seems to increase the risk of HCC[1,11]. Finally, there are synergistic effects between the different risk factors for HCC. For example, co-transfection with HCV and HBV has an additive effect on the HCC incidence, as does hepatitis virus infection plus AFB1 exposure or hepatitis virus infection plus chronic alcoholism.

Genetically modified mice, including transgenic, knock-out and knock-in mice, are able to provide animal models that help to elucidate the molecular mechanisms related to pathogenesis and carcinogenesis in the liver; furthermore, they also help us to evaluate potential chemopreventive agents and new therapeutic targets under physiological conditions. Although mice cannot be infected with HBV due to the narrow host range of HBV and the lack of a proper receptor or other factors for HBV, one of the advantages of using mouse models is the numerous and powerful resources that genetically modified mice make available. In this review, we mainly focus on the application of genetically modified mouse models to obtain a better understanding of HBV-associated carcinogenesis, the potential involvement

of tumor suppressor genes in HCC development, and the potential implications of these findings in mice to translational medicine.

HBV AND HCC DEVELOPMENT

HBV is one of the most important risk factors for HCC[1,3,4]. Clinically, chronic infection with HBV is highly associated with the incidence of HCC and with mortality. Additionally, the geographical distribution of liver cancer seems to be highly correlated with the prevalence of HBV in a population[1]. Epidemiological studies have identified areas with a low prevalence of HBV infection, such as in North America, Western Europe and Northern Europe; areas with an intermediate prevalence of HBV infection, such as Eastern and Southern Europe; and areas with the highest prevalence of HBV infection such as East and Southeast Asia and sub-Saharan Africa[1,3]. Additionally, the epidemiological results show that in the high HBV prevalence areas there are notable differences in the mean age of diagnosis of HCC between men and women, with men showing a lower mean age of diagnosis, and in the incidence of HCC among men than women, with men showing a higher incidence[1]. The age at which infection occurs, as well as environmental and dietary factors, all seem to be related to HCC incidence[1,3]. Infection with HBV is divided into two types, acute and chronic. Most adults with an acute infection of HBV (90% of cases) spontaneously recover and develop protective immunity[12-14]. HBV chronic infection in the remaining 10% of adults can be subdivided into four phases; these are the immune tolerance phase, the immune clearance [hepatitis Be antigen (HBeAg)-positive chronic hepatitis] phase, the inactive (carrier) phase and the reactivation (HBeAg-negative chronic hepatitis) phase; all of these phases may not all be seen in a given individual patient[15]. Up to 30% of HBV carriers develop chronic hepatitis, fibrosis, cirrhosis and eventually HCC[16,17]. In an area with high HBV prevalence, chronic HBV infection often occurs during birth (mother-child transmission). Therefore, an early onset of HBV infection and long-term chronic infection-induced advance liver diseases are likely to be the main factors contributing to the high mortality and high incidence of HCC in these regions[1]. Taiwan, which is located in an HBV high-prevalence region, was a pioneer of national HBV vaccination in the 1980s. A recent study has revealed that chronic disease mortality, HCC incidence and HCC mortality have significantly declined in both males and females who have received HBV immunization[18]. Nonetheless, the life-long effects of HBV vaccination will need to be observed for decades to come. Although immunization against HBV is highly efficacious in terms of preventing HBV-related liver disease, it also results in mutations affecting the genomes of HBV in circulation[19]. In addition, there is still no effective treatment that is able to eliminate HBV from infected patients[12].

HBV is a hepatocyte-specific enveloped DNA virus that is composed of large, middle and small surface proteins. Inside the envelope of the virus, the genome of HBV is packaged within the core proteins, which is formed into nucleocapsids. The genome of HBV is a 3.2-kb partially double-stranded circular DNA that is reversely transcribed from a 3.2-kb pre-genomic RNA (pgRNA). A receptor (sodium taurocholate co-transporting peptide, NTCP), which is responsible for HBV entry into host cells, was recently identified[20,21]. After entering the host cell, the nucleocapsids transport the HBV genome DNA into the nucleus of the infected hepatocytes. The circular HBV DNA is then converted into covalently closed circular DNA (cccDNA), forming a minichromosome inside the nucleus, and these minichromosomes serve as the template for RNA transcription. In some cases, the HBV genome has been found to integrate into the host genome of a patient with chronic HBV infection; however, this is not necessary for viral replication unlike the situation with retroviruses[14,22]. The HBV polymerase, which is covalently linked to the 5′ end of the negative strand of the genome, uses the 5′ end of oligoribonucleotides on the plus strand for reverse transcription. There are four promoters and a shared polyadenylation signal in the genome that are involved in regulating the transcription of HBV proteins. The core promoter is responsible for transcripts of the pgRNA (3.5 kb) and of the pre-core protein, whose transcript is slightly longer than that of the pgRNA. The pre-core protein is able to be secreted as HBeAg after removal of residues at the N-terminus and C-terminus. In addition, in order to serve as a template for reverse transcription, the pgRNA also encodes the core and polymerase proteins. The core protein, which is also known as hepatitis B core antigen, assembles the nucleocapsids of the virus and is responsible for delivering the viral nucleocapsids into the host nucleus. There are two promoters for the surface proteins, which generate 2.4-kb and 2.1-kb transcripts. The 2.4-kb transcript encodes the large surface protein, while the 2.1kb encodes the middle and small surface proteins. These three surface proteins (HBsAg) are synthesized in the rough endoplasmic reticulum (ER) and then transported to the Golgi apparatus for glycosylation; these proteins are then able to be secreted as non-infectious subviral particles. The smallest transcript (0.9 kb) is the template for the X protein. During viral replication, the core protein packages the 3.5-kb pgRNA and polymerase into nucleocapsids. Then, the reverse transcription of pgRNA is carried out inside of the nucleocapsid; this is carried out by the viral polymerase and generates the minus-strand of the HBV DNA first. The minus strand is then used as a template for the synthesis of the plus-strand of HBV DNA. Finally, the nucleocapsids are enveloped by surface proteins during processing on the ER and Golgi apparatus, which is followed by secretion of the virus from the cell[14,23,24].

GENETICALLY MODIFIED MOUSE MODELS USED TO STUDY HBV

The host range of HBV is highly limited and is restricted to humans and higher primates[23]. Early studies of hepatitis virus were carried out using woodchuck hepatitis virus (WHV), duck HBV (DHBV) and ground squirrel hepatitis virus (GSHV)[23]. However, the outbred properties of the hosts of WHV and DHBV limited our understanding of the specific molecular mechanisms involved in host-virus interaction and carcinogenesis. Previous studies revealed that the transgenic mouse carrying the HBV genome is not an ideal animal model to investigate the mechanisms of HBV infection. First, the viruses produced by the transgenic liver cannot infect and re-enter their own hepatocytes in mice. Second, the cccDNA, which is the template for viral transcription in humans, was undetectable in the livers of HBV transgenic mice. Third, the transgenic mice have immune tolerance to HBV therefore cannot develop hepatitis. Currently the chimpanzee and tupaia are the only animal models that can be infected by HBV. However, their use is limited by ethics, large size, and low feasibility as genetic manipulation model systems as well as by a very high cost; this compromises their utility in research and development[25]. The problem of establishing a mouse model which can be infected by HBV may be overcome by introducing the receptor of HBV and its critical domain for host-specificity of infection into the hepatocytes of mice by transgenic technology[20,21].

Although the mouse cannot be naturally infected by HBV, transgenic mice carrying the HBV genome have helped scientists to gain insights into the molecular mechanism of viral replication and assembly, and to serve as animal models for evaluation of anti-HBV therapy. First, the viral particles generated in transgenic mouse liver are highly similar to those in human patients. In addition, viruses purified from the blood of HBV transgenic mice can infect human fetal hepatocytes *in vitro*[26]. This indicated that the molecular mechanism regulating the synthesis of HBV transcripts and proteins, as well as the viral package and viral secretion, probably is shared in human and mouse hepatocytes. Accordingly, these transgenic mouse models may serve as an *in vivo* platform of animal models to evaluate therapeutic agents against viral replication by nucleoside analog or small interfering RNA[25]. Second, the HBV particles can be generated in transgenic liver but cannot re-enter mouse hepatocytes; this suggests mechanistically that mouse hepatocytes lack a proper HBV receptor for viral internalization. Third, the replication and expression of the HBV genome is not cytopathic or toxic to the host cells; there was no sign of hepatocarcinogenesis at a late stage of mouse life[26]. Notably, the results obtained from HBV transgenic mice were consistent with those from infection experiments of HBV in chimpanzee[14,27]. Thus, it seems that transgenic mice carrying the HBV genome are capable of recapitulating several aspects of the post-infected hepatocytes in natural hosts such as the human and chimpanzee.

There have been three possible mechanisms proposed for HBV-mediated HCC. These are: (1) that a viral protein *per se* is oncogenic; (2) that there is an infection-promoted immune response and this triggers a long-term process of carcinogenesis in liver; and (3) that the integration of HBV DNA affects the integrity of the host genome[10]. Researchers have tried to use small animals such as the mouse, rather than primates with a longer lifespan, in order to establish platforms for the study *in vivo* of mechanisms related to liver carcinogenesis. In 1985, two transgenic mouse lines expressing the HBV surface proteins were independently established by two different groups using genetically modified transgenic technology[28,29]. Later, transgenic mice carrying the core gene, the X gene and even the whole genome of the HBV were created. These transgenic mouse models have helped enhance our knowledge of HBV-related biology and liver pathogenesis (Table 1).

Mouse models for the HBV surface protein

In patients with HBV chronic infection, the surface protein is the dominant viral product and can be considered to be an onco-protein[28]. Aiming to mimic the human HBV carrier status, transgenic mice expressing the surface protein were the earliest HBV-related transgenic mice to be created[28,29]. One early and important observation was that the accumulation of filamentous surface protein in endoplasmic reticulum leads to hepatotoxicity and carcinogenesis[22,30-32]. The pathogenic effect of HBV surface proteins on the mouse liver are relevant to human HCC development in HBV carriers because phenomena such as ground glass cell formation are found[22,31,33,34]. The sexual dimorphism found to affect HCC incidence in humans can also be observed in transgenic mice[22,35-37]. Another research group generated HBsAg and HBV X protein (HBx) transgenic mice by the knock-in technique in order to reduce the effects of the random integration events that occur with conventional microinjection, and this model system also concluded that the HBV surface protein is oncogenic[37]. HBV vaccine is effective at decreasing HCC prevalence; however, mutated forms of the HBVs in circulation have emerged rapidly, particularly in the PreS/S region[38]. Clinically, patients harboring the PreS mutation are more susceptible to liver cirrhosis and HCC[39]. Efforts were later made to create a transgenic mouse that would help our understanding of the role of the mutated surface protein in pathology. These studies showed that the unfolded protein response (ER stress) is a major factor in HBV surface protein-induced carcinogenesis[33,40].

Table 1 Hepatitis B virus-related transgenic mice

Transgene	Promoter	Expression	Pathology	Ref.
PreS/S/X	Endogenous	Surface proteins	Until 6 mo: no obvious pathology	[28]
PreS/S/X (two TGs)	Endogenous and mouse metallothionein I	Surface proteins	Not determined	[29]
PreS/S/X	Mouse metallothionein I and Albumin	Surface proteins	Ground glass 7-9 mo: adenoma 12 mo: HCC 18 mo: HCC (100%)	[22,30-32,34]
Genome (2X)	Endogenous	Surface proteins	Not determined	[241]
PreS/S (Knock-in)	Mouse p21	Surface proteins	15-24 mo: HCC [53% (♂KI/+), 72% (♂KI/KI), 0% (♀KI/+ and KI/KI)]	[37]
PreS/S	Endogenous	Surface proteins	HCC	[33,40]
X	Human α-1-antitrypsin	HBx protein	Focal necrosis, hyperplasia nodule (Authors claimed that X is not tumorigenic)	[42]
X	Endogenous	HBx protein	4 mo: altered hepatocyte in multifocal area 10 mo: tumor nodule 16 mo: tumor (80%) 24 mo: HCC (84%)	[43,44]
X	Endogenous	HBx protein	6 mo: neoplastic nodules (66.6%) 15-18 mo: small neoplastic nodule (100%) and HCC (75%)	[41]
X (Knock-in)	Mouse p21	HBx protein	15-24 mo: HCC [60%-64% (♂KI/+ and KI/KI), 43%-46% (♀KI/+ and KI/KI)]	[37]
X	Mouse albumin	HBx protein	12 mo: dysplasia nodule (100%) 16 mo: HCC (80%) 20 mo: HCC (90%-100%)	[45-47]
Precore/core	Mouse metallothionein I	Precore protein	Not determined	[56]
Precore/core	Endogenous	Core protein	12 mo: no hepatitis or HCC	[13,57]
Precore/core (two TGs)	Endogenous	Precore and core proteins	Not determined	[58]
Genome (2X)	Endogenous	Replicative intermediates	10 mo: no obvious pathology	[60]
Genome (1.2X)	Endogenous	Replicative virus	12 mo: no hepatitis or HCC 24 mo: no obvious pathology	[59,61]
Genome (1.3X)	Endogenous	Replicative intermediates	Until 12 mo: no obvious pathology	[62]

PreS/S: Coding region of hepatitis B virus (HBV) large, middle and small surface proteins; X: Coding region of HBV X protein; Precore/core: Coding region of HBV pre-core/core proteins; KI: Knock-in allele; TG: Transgenic mice; Genome (2X): Indicates 2 copies of the complete 3.2-kilobase HBV genome.

Mouse models for the HBx

It has long been suggested that HBx plays a role in hepatocarcinogenesis because infection with DHBV, which lacks the X gene, does not result in the development of HCC, while infection with WHV and GSHV, which do have the X gene, does result in host HCC[41]. In addition, the X gene is highly conserved between different HBV subtypes[42]. Interestingly, the X transcript is the most abundant mRNA compared to other HBV transcripts in some clinical specimens of human HCC[41]. Importantly, several independently generated HBx transgenic mouse models have demonstrated that HBx alone is able to induce malignant transformation of hepatocytes[37,41-47] (Table 1).

The functions of the HBx protein have been extensively studied, particularly in cell culture systems, and are known to involve multiple cellular events, including transactivation of transcription factors, cell cycle progression, several signaling transduction pathways, mitochondrial homeostasis, cell death, DNA instability, glucose metabolism and lipid metabolism[4,10]. It is possible, using transgenic mice, to test *in vivo* whether the above HBx-mediated cellular events indeed are involved in hepatocarcinogenesis[4]. In addition, these models allow the physiological interacting partners of HBx to be evaluated. Other than mouse, various other model organisms have been used for this type of *in vivo* studies, for example *C. elegans*[48]. These studies have shown that the HBx protein binds to CED-9, which is a homolog of members of the anti-apoptotic Bcl-2 protein family; this binding triggers cell death in *C. elegans* and in a human hepG2 cell line[48,49]. Interestingly, these studies also discovered that when HBx is mutated, it is unable to induce CED-9-regulated cell death. Further investigation of its role in carcinogenesis using mouse models will help our understanding of the molecular mechanism of HBx-mediated HCC.

Previously, we have generated four lines of HBx transgenic mice, namely A105, A106, A110 and A112, using the C57BL/6 background[46]. All of the HBx transgenic lines generated in our laboratory spontaneously develop HCC at 13 to 16 mo of age. At the patho-histological level, the HCC developed in the HBx transgenic mice exhibits a well-differentiated morphology involving the trabecular pattern. Fibrosis, bizarre nuclei, cytoplasmic lipid droplets and hyaline globules can be observed in the HBx-induced HCC

samples, which is similar to the situation observed in human HCC samples[46]. Furthermore, a gender disparity for HCC in the HBx transgenic mice has also been observed[37,43,46]. The HBx transgenic mice thus provide an animal model for mechanistic studies[45,50-53] and for the evaluation under physiological conditions of new chemopreventive agents and new therapeutic agents for HCC[47,54,55].

At the ultrastructural level, we have carried out transmission electron microscope (TEM) examinations and these have revealed that there are alterations in the organelles of the hepatocytes of the HBx transgenic mice (Figure 1). In wild-type mice, each normal hepatocyte typically contains a spherical nucleus with homogeneous euchromatin and heterochromatin. Long and stacked cisternae of rough endoplasmic reticulum are located parallel to the nuclear envelope and the lateral edges of the cell. Numerous mitochondria of varying lengths are present, and these range in shape from spherical mitochondria to dumbbell and rod-like shaped mitochondria. Hepatocellular glycogen is apparent in the electron micrographs as irregular non-membrane bound and rosette structures (Figure 1A and A'). In the HBx transgenic mice, TEM reveals that a dramatic decrease has occurred in the density of cytoplasmic organelles (Figure 1B-D). Many ultrastructural abnormalities of the mitochondria can be detected and these include breakdown and swelling of mitochondrial membranes, as well as an increase in the electron density of the matrix (Figure 1B and B'). In addition, the phagophore isolation membrane (PIm), which is the result of the endoplasmic reticulum engulfing degenerated mitochondria, is now present, which leads to the formation of autophagosomes (Figure 1B and B'). The endoplasmic reticulum is dilated or forms a structure with irregular fragmentation and degeneration (Figure 1C and C'). Part of the degenerated membrane debris of mitochondria and endoplasmic reticulum can be detected as irregular concentric myelin figures (Figure 1D and D'). Furthermore, some hepatocytes of HBx mice have abundant peroxisomes; this is likely to be due to the oxidative stress induced by the HBx protein (Figure 1D and D'). Moreover, the nuclear envelopes of some hepatocytes of HBx transgenic mice have undergone breakdown (Figure 1D and D'; Figure 1C and C'). All of these ultrastructural alterations and organelle degeneration events, particularly the abnormalities in the mitochondria and endoplasmic reticulum, appear at a very early stage (4-8 wk old) and contribute to the progression of carcinogenesis in the livers of HBx transgenic mice.

Mouse models for the HBV precore/core protein and the whole genome

Previously, several groups have established transgenic mice in order to investigate HBV assembly within the hepatocyte *in vivo* (Table 1). Transgenic mice carrying the precore or core proteins of HBV have no overt phenotype (no sign of hepatitis and HCC formation) up to 12 mo-old[13,56-58]. Transgenic mice carrying the whole HBV genome have also been generated in order to study the HBV life cycle and its interaction with host factors[26,59-62] (Table 1). Virus particles that contain HBsAg, HBc/eAg and HBV DNA can be detected in the bloodstream of these transgenic mice. Interestingly, a gender difference in the synthesis of HBeAg and intermediates of the replicative virus can be observed in these mice[63]. However, similar to the precore/core transgenic mice, there are no obvious pathological changes detectable in the transgenic liver. Nevertheless, mice carrying the whole HBV genome do provide animal models that are amenable to the study of the HBV life cycle, and they can also help the screening of therapeutic agents against HBV replication.

CONCLUSION AND PERSPECTIVES IN RELATION TO THE HBV-RELATED MOUSE MODELS

No single transgenic mouse model is able to cover all aspects of HCC pathogenesis. Currently there are still some barriers when using genetically modified mice, such as their inbred genetic backgrounds versus the complications of human genetic variation, the inability to be infected by HBV, the lack of an immunopathogenesis process, the scarcity of cirrhosis, and the very rare nature of spontaneous metastasis. Accordingly, alternative strategies have been created and applied to mice in order to study HBV biology. For example, hydrodynamic injection of HBV DNA into mice[25,64] and humanized mice[65]. These models have further extended the feasibility and have helped to create an understanding of virus-host interactions as well as the pathological effects of HBV mutant proteins.

Transgenic mice carrying the HBV genome or expressing various HBV proteins provide valuable models that help to elucidate clinic observations, allowing mechanistic investigations to be performed and helping to test relevant hypotheses. These approaches also help our understanding of HBV replication and the regulation of HBV gene expression, as well as of the inflammation and innate immune response that is induced by HBV proteins under physiological conditions[13,63,66-68]. In addition, these previously published transgenic mouse models have been employed as a platform for discovery of therapeutic targets and/or for the identification of preventive chemicals[4,27,47,54,55,69]. For example, novel HBV-related microRNAs present in the hepatic and general circulation have been discovered over the past decades and are considered to be potential targets for therapy[10,70]. The possible roles of these microRNAs in carcinogenesis and their therapeutic applications can certainly be tested using the available established HBV-

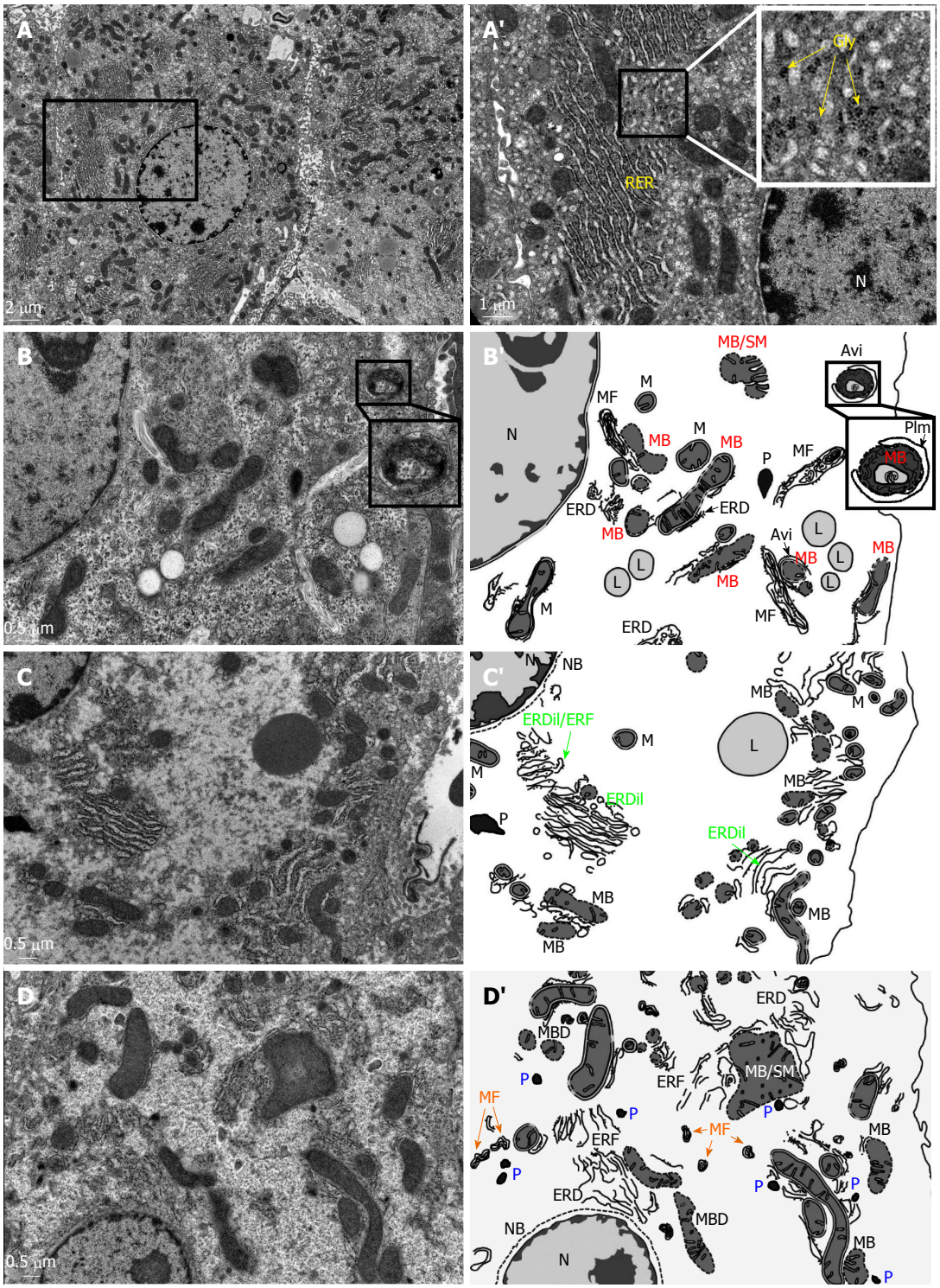

Figure 1 Ultrastructural alterations in the hepatocytes of the hepatitis B virus X protein transgenic mice revealed by transmission electron microscope. A: Ultrastructure of the hepatocyte of a wild-type mouse (2-mo old). A': the inset in A was enlarged to provide a better perspective of intact mitochondria (M), rough endoplasmic reticulum (RER), rosette glycogen (Gly) and nucleus (N). B-D: In the HBx transgenic mice (2-mo old), severe ultrastructural alterations can be observed in the hepatocytes. These include nuclear envelope breakdown (NB), a significant decrease in the density of cytoplasmic organelles, abundant peroxisomes (P), the disorganization of rough endoplasmic reticulum [*e.g.*, endoplasmic reticulum fragmentation (ERF), endoplasmic reticulum dilation (ERDil), and/or endoplasmic reticulum degeneration ERD)], mitochondria membrane breakdown (MB), swollen mitochondria (SM), the presence of lipid droplets (L), and appearance of myelin figures (MF), which are the membranous debris of mitochondria or ER degeneration. B'-D': Schematic presentation of the ultrastructural alterations in the HBx hepatocytes shown in panel B-D. The inset in B and B' provides a better perspective of a phagophore isolation membrane (Plm) engulfing a degenerated mitochondrion leading to the formation of autophagosomes (AVi).

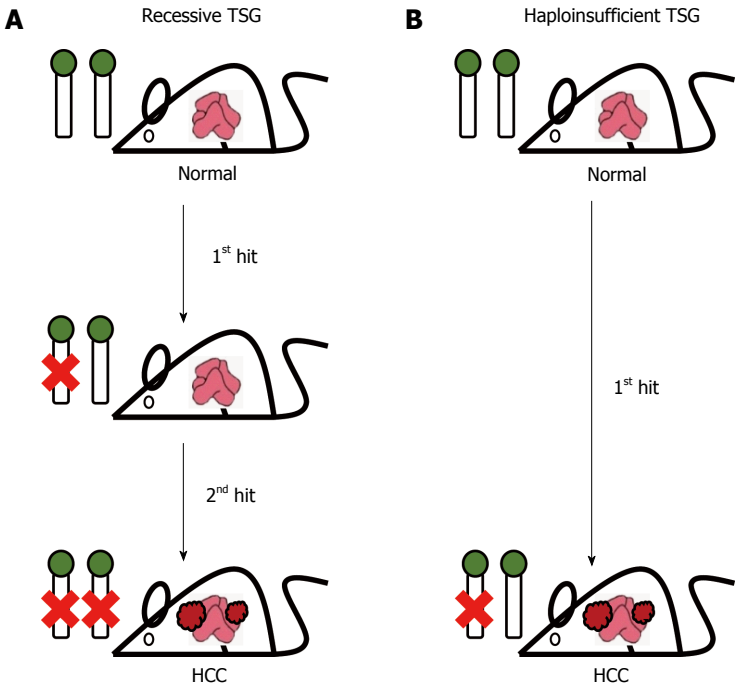

Figure 2 Tumor suppressor genes in hepatocellular carcinoma. A: Recessive tumor suppressor genes (TSGs) need there to be loss-of-function *via* two hits (mutation or genetic modification) in order to trigger tumor formation. Only when there is homozygous deficiency of a recessive TSG does this lead to hepatocellular carcinoma (HCC) development; B: Haploinsufficient TSGs are functionally insufficient to suppress tumor development when there is loss of only a single allele. Heterozygous deficiency of a haploinsufficient TSG can cause HCC development in the liver even when the second allele remains intact.

related mouse models. The ultimate goal, of course, is still to use transgenic mice to find a cure for patients with HBV infection and HCC. Nevertheless, there is still a great need for a good animal model for HCC and such a model would be expected to meet as many of the following requirements as possible. These are: (1) the faithful reproduction of HCC's progression stages and incidence; (2) a reliable reproduction of the molecular and cellular events during HCC carcinogenesis; (3) the ability to mimic the tumor-host and tumor-metastases interactions; (4) a reproduction of the tumor micro-environment in human patients; and (5) the ability to manipulate in a feasible manner the genome of HBV[4].

Additionally, the HBV virus at present is also evolving in response to environmental changes such as vaccination or drug administration. For example, the emergence of mutated hepatitis B surface protein, which may potentially have vaccine-resistant properties, is a serious issue[38]. The generation of new transgenic mouse model systems is one way to dissect the effects of such mutant HBV proteins on liver carcinogenesis. Lately, a newly developed genome-editing tool, the Crips/Cas9 system, has been successfully applied and used to target the HBV DNA and reduce the production of HBV proteins[71-73]. Using a hydrodynamics-HBV persistence mouse model, Lin *et al*[72] have demonstrated that the Crips/Cas9 system is able to cleave the intrahepatic HBV genome containing the plasmid and facilitate its clearance in animals. These findings suggest that the genome-editing tool may have potential in the eradication of persistent HBV infection.

TUMOR SUPPRESSOR GENES DURING HCC DEVELOPMENT

The identification of tumor suppressor genes (TSGs) is very important to the development of novel therapeutic strategies targeting HCC. Many TSGs have been identified in human HCC tissue samples, using cell culture systems and with mouse models[74-77]. These TSGs can be classified into two major groups, the recessive TSGs and the haploinsufficient TSGs (Figure 2). The recessive TSG group follows the Knudson's two-hit hypothesis, in which a deficiency affecting both alleles of a TSG triggers HCC development. For the haploinsufficient TSG group, the loss of a single allele of a TSG is sufficient to promote tumorigenesis, with the second allele remaining intact in the tumors. These latter TSGs are functionally haploinsufficient in the heterozygous condition and it would seem that cellular level of 50% for their gene product is insufficient to suppress tumor formation[78-80]. Using mouse models, scientists have been able to delineate the molecular mechanisms underlying the roles of both recessive and haploinsufficient TSGs during the suppression of carcinogenesis in liver. These findings should contribute significantly to the development of novel chemopreventive or therapeutic strategies for HCC. Here, we will review the TSGs in HCC mainly based on the pathways in which they are involved (Table 2).

Table 2 Mouse models of tumor suppressor genes in hepatocellular carcinoma

Official gene name (common name)	Mouse genotype	Mouse genetic background	Pathology	TGS type	Functions	Human Chromosome location	Expression in human HCC tumor	Ref.
Autophagy regulator								
Becn1 (Beclin1)	*Beclin1*$^{+/-}$	Mixed (129Sv/J; C57BL/6J)	16-18 mo: HCC (8%) 18-22 mo: HCC (26%) Promote HBV-mediated HCC	Haplo-insufficiency	Regulation of autophagy	17q21	Down	[85-87]
Atg5	*Alb-Cre*; *Atg5*$^{flx/flx}$	C57BL/6	2 mo: hepatic cell death, proliferation, inflammation, fibrosis and hepatomegaly 12 mo: hepatic adenoma (100%)	Recessive	Control autophagosome formation	6q21	Down	[90,91]
Atg7	*Alb-Cre*; *Atg7*$^{flx/flx}$;	C57BL/6	4 mo: fatty liver 12 mo: hepatic adenoma (90.9%)	Recessive	Control autophagosome formation	3p25.3	Up	[92-94]
Cell cycle regulator								
Klf6	*KLF6*$^{+/-}$	C57BL/6	Promote DEN-induced HCC	Haplo-insufficiency	Cell cycle regulation	10p15	Down	[99,101]
Plk4	*Plk4*$^{+/-}$	Mixed (129Sv;CD1)	3 mo: mitotic failure in hepatocyte regeneration 18-24 mo: HCC (30%)	Haplo-insufficiency	Control of cell cycle progression	4q28	Down	[104,105]
Cdkn1a (p21)	*p21*$^{+/-}$	Mixed (129Sv; C57BL/6)	Promote γ-irradiation-induced HCC	Haplo-insufficiency	Inhibits the activity of CDK2 or CDK4	6p21.2	Up	[107,242]
	p21$^{-/-}$	C57BL/6	Promote HCC in Nemo liver-specific KO	Recessive				[108]
Trp53 (p53)	*p53*$^{+/-}$	C57BL/6	Promote HBV-mediated and AFB1-induced HCC	Haplo-insufficiency	DNA damage response transcription factor	17p13.1	Up	[111,243]
	Aflp-Cre; *p53*$^{flx/flx}$	C57BL/6	14-20 mo: HCC (90%)	Recessive				[112]
Protein ubiquination								
Amfr (Gp78)	*Gp78*$^{-/-}$	C57BL/6	12 mo: fatty liver, inflammation and HCC (24%)	Recessive	E3 ubiquitin ligase	16q21	Down	[117]
Park2 (Parkin)	*Parkin*$^{-/-}$	Mixed (129/C57BL6SJL)	11-12 mo: hepatocyte proliferation and hepatomegaly 16-17 mo: HCC (33%) 22-23 mo: HCC (45%)	Recessive	E3 ubiquitin ligase	6q25.2-q27	Down	[118,122]
Trim24	*Trim24*$^{-/-}$	C57BL/6	2 mo: hepatic cell death and inflammation 4-6 mo: fatty liver, fibrosis and liver nodule 9-21 mo: HCC (55%)	Recessive	E3 ubiquitin ligase	7q32	Up	[119,125]
Genome stability maintenance								
Anxa7 (Annexin 7)	*Anx7*$^{+/-}$	C57BL/6	10 mo: Hepatomegaly 12 mo: HCC (3.6%)	Haploinsufficiency	Ca^{2+}-dependent endocrine secretion	10q22.2	Up	[131,132]
Nbn (Nibrin)	*Nbn*$^{+/-}$	Mixed (129Sv; C57BL/6)	23 mo: HCC (8.6%)	Haploinsufficiency	DNA repair	8q21	Up	[134,244]
Pinx1	*PinX1*$^{+/-}$	Mixed (129Sv; C57BL/6)	9-18 mo: HCC (17.7%)	Haploinsufficiency	Telomerase inhibitor	8p23	Down	[136,137]
Sgol1 (Shugoshin 1)	*Sgo1*$^{+/-}$	C57BL/6	4 mo: hepatocyte DNA damage 12 mo: HCC Promote AOM-induced HCC	Haploinsufficiency	Protector of chromosome cohesion and centrosome integrity	3p24.3	Up	[139,140]
Metabolic function								
Acox1 (Acyl-CoA oxidase 1)	*Aox*$^{-/-}$	Mixed (129/Ola; C57BL/6)	2-4 mo: fatty liver, inflammation and hepatocyte proliferation 10-15 mo: HCC (100%)	Recessive	Peroxisomal β-oxidation	17q25.1	Not determined	[144]

Bhmt	*Bhmt*$^{-/-}$	C57BL/6	1 mo: fatty liver 12 mo: HCC (50%)	Recessive	Methionine metabolism	5q14.1	Down	[149,150]
Nr1h4 (Farnesoid X receptor)	*Fxr*$^{-/-}$	C57BL/6N	3 mo: hepatic cell death and proliferation 6-9 mo: fatty liver and inflammation 12 mo: fibrosis and HCC (16.1%)	Recessive	Transcriptional regulation of synthesis and transport of bile acids	12q23.1	Down	[155,157]
Gnmt (Glycine N-methyltransferase)	*Gnmt*$^{-/-}$	Mixed (129Sv; C57BL/6)	3 mo: fatty liver and fibrosis 8 mo: HCC (100%) at 8-mo	Recessive	Methionine metabolism	6p12	Down	[148,151]
Abcb4 (Mdr2)	*Mdr2*$^{-/-}$	129/OlaHsd	3 mo: inflammation and fibrosis 6-mo: HCC	Recessive	Phosphatidylcholine translocase	7q21.1	Down	[156,158]
Hippo signaling pathway								
Stk4 (Mst1)	*Alb-Cre*; *Mst1/2*$^{flx/flx}$	Mixed (129Sv; C57BL/6;CD1)	1 mo: hepatocyte proliferation and hepatomegaly 6 mo: HCC (100%)	Recessive	Kinase (negative regulator)	20q11.2	Not determined	[165,166]
Stk3 (Mst2)	*Alb-Cre*; *Mst1/2*$^{flx/flx}$	Mixed (129Sv; C57BL/6;CD1)	1 mo: hepatocyte proliferation and hepatomegaly 6 mo: HCC (100%)	Recessive	Kinase (negative regulator)	8q22.2	Not determined	[165,166]
Nf2 (Neurofibromin 2)	*Alb-Cre*; *Nf2*$^{flx/flx}$	Mixed (FVB/N; C57BL/6)	2 mo: oval cell over-proliferation and hepatomegaly 7 mo: HCC (100%)	Recessive	Negative regulator	22q12.2	Not determined	[168]
Jak/Stat signaling pathway								
Socs1	*Socs1*$^{+/-}$	C57BL/6	Promote DEN-induced HCC	Haploinsufficiency	Negative regulator	16p13.13	Down	[174]
Socs3	*Socs3*$^{+/-}$	Mixed (129Sv; C57BL/6)	Promote DEN-induced HCC	Haploinsufficiency	Negative regulator	17q25.3	Down	[176]
Ptpn11 (Shp2)	*Alb-Cre*; *Shp2*$^{flx/flx}$	C57BL/6	2-3 mo: inflammation and hepatic cell death 8-mo: hyperplasia nodule 12-18 mo: hepatic adenoma (68%) Promote DEN-induced HCC	Recessive	Protein tyrosine phosphatase (negative regulator)	12q24	Down	[179]
Ptpro	*Ptpro*$^{-/-}$	C57BL/6	Promote DEN-induced HCC	Recessive	Receptor-like protein tyrosine phosphatase (negative regulator)	12p13.3	Down	[181,182]
NF-κB signaling pathway								
Cyld (Cylindromatosis)	*Alfp-Cre*; *Cyld*$^{flx/flx}$	C57BL/6 congenic	1-2 mo: hepatic cell death, inflammation and fibrosis 12 mo: HCC	Recessive	Deubiquitinase (negative regulator)	16q12.1	Down	[188,189]
Lgals3 (Galectin-3)	*Gal3*$^{-/-}$	CD1	6 mo: fatty liver and inflammation 15 mo: fibrosis and liver nodule 25 mo: HCC (100%)	Recessive	Regulation of inflammatory responses	14q22.3	Up	[192-194]
Ikbkg (Nemo)	*Alfp-Cre*; *Nemo*$^{flx/flx}$	C57BL/6	2 mo: fatty liver, inflammation, hepatic cell death and proliferation 6 mo: dysplastic nodule 12 mo: HCC (100%)	Recessive	Activation of NF-κB	Xq28	Down	[195,196]
Map3k7 (Tak1)	*Alfp-Cre*; *Tak1*$^{flx/flx}$	Mixed (129/Ola; C57BL/6)	1-2 mo: hepatic cell death, proliferation and fibrosis 4-8 mo: HCC (88%)	Recessive	Serine/threonine protein kinase (Activation of the NF-κB)	6q15	Not determined	[197]
PI3K/Akt/mTOR signaling pathway								
Stk11 (Lkb1)	*Lkb1*$^{+/-}$	Mixed (129Sv; C57BL/6)	10-12 mo: Hepatic hyperplasia, HCC (29%) 14 mo: HCC (75%)	Recessive	Serine/threonine kinase (activator of AMPK)	19p13.3	Down	[201,202]

Pten	*Alb-Cre*; *Pten*$^{flx/flx}$	C57BL/6	2-3 mo: fatty liver and hepatomegaly 9-10 mo: inflammation and fibrosis 17-18 mo: HCC (66%)	Recessive	Phospholipid phosphatase (negative regulator)	10q23.3	Down	[204,208]
Raptor (Regulatory associated protein of mTOR, complex 1)	*Alb-Cre*; *Raptor*$^{flx/flx}$	C57BL/6	2 mo: hepatic cell death, inflammation and fibrosis Promote DEN-induced HCC	Recessive	Activation of mTOR activity	17q25.3	Up	[209,210]
TGF-β signaling pathway								
Sptbn1 (β-spectrin)	*Elf*$^{+/-}$	Mixed (129SvEv; Black Swiss)	15 mo: Fatty liver, HCC (40%)	Haploinsufficiency	Propagation of TGF-β signal	2p21	Down	[214]
Tgfb1 (TGF-β1)	*Tgf*-β1$^{+/-}$	C57BL/6NCr	Promote DEN-induced HCC	Haploinsufficiency	Growth factor	19q13.1	Up	[216,245]
Tgfbr2 (TGF-β type II receptor)	*TβR-II*$^{+/-}$	Mixed (129Sv; C57BL/6)	Promote DEN-induced HCC	Recessive	TGF-β receptor	3p22	Down	[217,218]
Wnt signaling pathway								
Apc	*Adenovirus-Cre*; *Apc*$^{flx/flx}$	C57BL/6N	9 mo: HCC (67%)	Recessive	Antagonist	5q21	Down	[222,223]
MicroRNA								
Mir122	*Mir122a*$^{-/-}$	C57BL/6	3 mo: fatty liver, inflammation and fibrosis 11 mo: HCC (75%)	Recessive	Post-transcriptional regulation of gene expression	18q21.31	Down	[227,228]
Mir140	*Mir140*$^{-/-}$	C57BL/6	Promote DEN-induced HCC	Recessive	Post-transcriptional regulation of gene expression	16q22.1	Not determined	[229]
Miscellaneous								
Ncoa5 (Nuclear receptor coactivator 5)	*Ncoa5*$^{+/-}$	Mixed (129Sv; C57BL/6)	6 mo: fatty liver 10 mo: inflammation and fibrosis 10-18 mo: HCC (94%)	Haploinsufficiency	Estrogen receptor coactivator	20q13.12	Down	[231]
Prkar1a	*Prkar1a*$^{+/-}$	Mixed (129Sv/J; C57BL/6)	9-19 mo: HCC (29.4%)	Haploinsufficiency	Regulation of the serine/ threonine kinase activity	17q24.2	Up	[233,246]
Ncoa2 (Nuclear receptor coactivator 2)	*Ncoa2*$^{-/-}$	Mixed (129Sv/J; C57BL/6)	Promote DEN-induced HCC	Recessive	Transcriptional coactivator	8q13.3	Down	[235]
Nfe2l1 (Nuclear factor, erythroid 2-like 1)	*Alb-Cre*; *Nrf1*$^{flx/flx}$	Mixed (129Sv; C57BL/6)	1-2 mo: fatty liver, necrosis and inflammation 6 mo: fibrosis 12 mo: HCC (100%)	Recessive	Transcription factor	17q21.3	Not determined	[238]

HCC: Hepatocellular carcinoma.

TSGS AND HCC MOUSE MODELS

Autophagy regulatory genes

Autophagy is a highly regulated process that degrades damaged cellular organelles and macromolecules allowing recycling of bioenergetic molecules. Many proteins are involved in the autophagy pathway, including the Beclin1 and autophagy-related gene (Atg) proteins. Autophagy plays dual roles in hepatocarcinogenesis; firstly, it acts as a tumor suppressor during the initiation stages of HCC, while exerting a tumor supportive function during the promotion and progression stages. In normal cells, autophagy has a tumor suppressor function that aims to maintain normal metabolism, preserve genetic stability and inhibit inflammation. On the other hand, autophagy also plays a role in supporting tumor progression by helping cancer cells survive stress-induced cell death[81,82]. The tumor suppressor function of autophagy has been demonstrated using mouse models.

Beclin1: Beclin1 is one of the major mediators of autophagy; this gene is located on human chromosome 17q21. Mono-allelic deletion has been detected in various human cancers including ovarian, breast and prostate cancers[83]. A decrease in the levels of autophagy has been observed in a highly malignant HCC cell line compared with immortalized normal hepatic cells. Furthermore, the expression level of Beclin1 has been found to be decreased in human HCC tissues compared with the adjacent non-tumor

tissues[84,85]. Homozygous knockout of *Beclin1* leads to embryonic lethality; this finding demonstrates that Beclin1 is essential for early embryonic development in mice. Importantly, heterozygous deficiency of *Beclin1* leads to the development of spontaneous HCC and accelerates HBV-induced hepatocarcinogenesis in mice. Moreover, the wild-type allele of *Beclin1* has been found not to be mutated/lost in the HCC tissue of the *Beclin1*$^{+/-}$ mice[86,87]. These studies demonstrated that *Beclin1* is a haploinsufficient TSG in relation to HCC.

ATG5 and ATG7: *Atg5* and *Atg7* are autophagy-related genes (Atg) that promote the elongation of the autophagosome membrane. Homozygous knockout of either *Atg5* or *Atg7* leads to impairment of autophagosome formation and postnatal lethality in mice[88,89]. Regarding ATG5, mutation and/or loss of ATG5 expression has been observed in human HCC tissues[90]. In mice, hepatocyte-specific knockout of *Atg5* was found to result in hepatomegaly, hepatic cell death, compensatory proliferation of hepatocytes, inflammation and fibrosis, as well as the development of hepatocellular adenomas in mice[91]. Regarding ATG7, the expression of the ATG7 protein is increased in human HCC tissue samples compared with their adjacent non-tumor tissue samples, but the mechanism is currently unknown[92]. However, mice with a hepatocyte-specific knockout of *Atg7* develop fatty liver and hepatocellular adenoma, indicating that autophagy is involved in lipid metabolism and plays a role in suppressing tumor formation in the liver[93,94].

Cell cycle regulatory genes

Cell cycle progression is regulated by cyclin-dependent kinases (CDKs) together with several activators (cyclins) and CDKs inhibitors (p21 and p27). In normal tissue, the cell cycle is carefully controlled and regulated in order to maintain cell number homeostasis[95]. Abnormal cell cycle progression and sustained cell proliferation is one of the hallmarks of cancer cells[96]. Many genes involved in cell cycle control have potential tumor suppressive roles in HCC.

Kruppel-like factor 6: Kruppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcription factor that regulates the cell cycle and signal transduction. KLF6 is frequently inactivated by loss of heterozygosity, somatic mutation or promoter methylation in various cancers including prostate, colon and liver cancer[97,98]. In mice, heterozygous deficiency of *Klf6* promotes diethylnitrosamine (DEN)-induced hepatocarcinogenesis in *Klf6*$^{+/-}$ mice; however, whether the wild-type allele of *Klf6* remained intact in the HCC was not determined during this study[99]. Liver-specific knockout of the two alleles of *Klf6* enhanced DEN-induced HCC in mice[100]. In humans, expression of KLF6 is decreased in HCV-related HCC tissue samples[99]. Down-regulation of KLF6 has also been observed in HBV-related human HCC[101]. These studies in humans and mice suggest that *KLF6* may function as a haploinsufficient TSG in HCC.

Polo-like kinase 4: Polo-like kinase 4 (PLK4) is a member of the polo-like kinase protein family that plays a critical role in cell cycle progression[102]. Homozygous knockout of *Plk4* resulted in embryonic lethality due to a mitotic defect in mice[103]. Importantly, heterozygous deficiency of *Plk4* leads to mitotic failure of regenerated hepatocytes and spontaneous HCC development in the heterozygous knockout mice. Furthermore, the wild-type allele of *Plk4* has been shown to remain intact in these HCC tissue samples[104]. In humans, the expression level of PLK4 is down-regulated in HCC tissue samples[105]. These studies indicate that *PLK4* functions as a haploinsufficient TSG in HCC.

p21: The p21 protein is an inhibitor of CDK and is involved in cell cycle control, cell senescence and cell death[106]. Heterozygous and homozygous knockout of *p21* promotes γ-irradiation-induced tumor formation in many types of cancers including HCC. The p21 protein has been found to be expressed in malignant tumors from irradiated *p21*$^{+/-}$ mice, suggesting that *p21* functions as a haploinsufficient TSG in this situation[107]. Moreover, homozygous knockout of *p21* in hepatocytes has been shown to enhance NEMO-mediated hepatocarcinogenesis in mice[108]. These results suggested that *p21* is able to function as a recessive or haploinsufficient TSG across various different tissues under a range of different physiological/environmental settings in mice[107].

p53: The p53 protein is a transcription factor that regulates DNA damage and stress responses. This protein functions as a tumor suppressor in many types of cancers[109,110]. Heterozygous knockout of *p53* has been shown to promote HBV-mediated and AFB1-induced HCC development in mice; furthermore, the wild-type allele of *p53* has been found to remain intact in the HCC tissues of these *p53*$^{+/-}$ mice, suggesting that *p53* functions as a haploinsufficient TSG in these circumstances[111]. Liver-specific knockout of the two alleles of *p53* leads to spontaneous HCC development in mice. These findings suggest that *p53*, like *p21*, is able to function as either a recessive TSG or a haploinsufficient TSG in different tissues under a range of different physiological/environmental settings in mice[112].

Protein ubiquitination

Intracellular protein degradation occurs *via* two major pathways; these are the ubiquitin-proteasome system and the lysosomal-mediated proteolysis pathway. The ubiquitin-proteasome system is a multi-step process involving protein labeling by polyubiquitination, which is followed by protein degradation *via* the proteasome.

Protein ubiquitination is a tightly regulated process that involves three major enzymes, namely E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase[113,114]. The E3 ubiquitin ligases act by targeting specific proteins, including gene products from oncogenes and tumor suppressor genes. Abnormal regulation of the E3 ubiquitin ligase is involved in the development of many types of cancer, including HCC[115,116]. Many E3 ubiquitin ligases have been demonstrated to function as tumor suppressors of HCC in mice, including Gp78, Parkin and Trim24[117-119]. These mouse models have been able to uncover the role of E3 ubiquitin ligases in hepatocarcinogenesis.

Glycoprotein 78: Glycoprotein 78 (GP78) is an endoplasmic reticulum (ER) membrane-anchored E3 ubiquitin ligase, which is involved in the ER-associated degradation (ERAD) pathway. The ERAD pathway is a protective mechanism that helps to maintain ER protein homeostasis through degradation of misfolded proteins[120]. In mice, homozygous knockout of *Gp78* results in the development of non-alcoholic steatohepatitis (NASH), liver fibrosis and HCC. In humans, GP78 has been shown to be down-regulated in HCC tissue samples compared to adjacent non-tumor liver tissue samples[117]. These studies suggest that *Gp78* is a regulator of ER homeostasis in the normal liver, and functions as a recessive TSG in HCC.

Parkin: Parkin is an E3 ubiquitin ligase involved in protein turnover, the stress response, mitochondrial homeostasis, metabolism and cell growth. Inactivation of Parkin has been frequently detected in many types of human cancers including HCC[121]. In humans, the protein levels of Parkin have been shown to be decreased in HCC tissue samples and HCC cell lines[122]. In mice, Parkin deficiency leads to hepatomegaly and HCC *via* an increase in the hepatocyte proliferation and a reduction in hepatic cell death. Additionally, disruption of Parkin results in impaired lipid uptake by hepatocytes when the mice are fed a high fat diet (HFD)[118,123]. These studies reveal that Parkin plays an important role in maintaining cell numbers and lipid homeostasis in the liver and functions as a recessive TSG in HCC.

Tripartite motif 24: Tripartite motif 24 (TRIM24) is located in the HCC critical region on human chromosome 7q32, suggesting that *TRIM24* may function as a tumor suppressor in HCC. TRIM24 is an E3 ubiquitin ligase, which negatively regulates the level of p53[124]. Upregulation of the TRIM24 protein has been observed in human HCC tissue samples compared with their adjacent non-tumor tissue samples[125]. Trim24 deficiency has been found to result in the development of fatty liver, hepatic injury and spontaneous HCC in homozygous knockout mice, which suggests that *Trim24* functions as a recessive TSG in HCC[119]. In addition, these studies have also revealed that the expression level of Trim24 is an important factor in the progression of HCC.

Genes related to genomic stability maintenance

Genomic stability is very important because it allows cells to avoid neoplastic transformation and tumorigenesis. The major mechanisms for maintaining genomic stability are high-fidelity DNA replication, accurate chromosome segregation, faithful DNA repair and cell cycle checkpoint control[126]. Genomic instability is a hallmark of most cancers and significantly contributes to cancer initiation and progression[127,128]. Many haploinsufficient TSGs in HCC are functionally involved in the maintenance of genomic stability.

Annexin 7: Annexin 7 (ANX7) is a member of the annexin family and is a Ca^{2+}-dependent phospholipid-binding protein[129]. Homozygous knockout of *Anx7* in mice leads to postnatal lethality at embryonic day 10 due to cerebral hemorrhage. Interestingly, heterozygous knockout of *Anx7* results in a Ca^{2+}-dependent endocrine secretory defect in mice, indicating that *Anx7* plays an important role in the regulation of endocrine secretion[130,131]. In addition, heterozygous deficiency of *Anx7* exhibits a phenotype of hepatomegaly and HCC develops spontaneously in these mice; the wild-type allele of *Anx7* has been found to remain intact in the HCC tissue from these $Anx7^{+/-}$ mice[132]. Genomic instability and downregulation of tumor suppressor genes have been observed in the HCC of $Anx7^{+/-}$ mice. These findings suggest that *Anx7* helps to maintain genomic stability and functions as a haploinsufficient TSG in HCC.

Nibrin: Nibrin (NBN) is involved in the repair of DNA double stranded breaks and has the potential to function as a tumor suppressor gene[133]. Homozygous knockout of *Nbn* causes embryonic lethality between embryonic day E3.5 and E7.5 in mice. Importantly, heterozygous deficiency of *Nbn* results in many types of spontaneous tumors including prostate cancer, mammary gland tumors, lymphoma and HCC in mice. Various chromosome aberrations, including fragmentation and end-to-end fusion, have been observed in the mouse embryonic fibroblasts (MEFs) of $Nbn^{+/-}$ mice. Loss of heterozygosity was found not to occur in the HCC tissues from the $Nbn^{+/-}$ mice[134]. These findings indicate that *Nbn* plays an important role in the maintenance of chromosome stability and would seem to function as a haploinsufficient TSG in HCC.

PIN2/TRF1-interacting telomerase inhibitor 1: PIN2/TRF1-interacting telomerase inhibitor 1 (PinX1) is a potent telomerase inhibitor involved in maintaining

telomeres at optimal length and its expression level is frequently down-regulated in the HBV-related HCC[135,136]. Heterozygous deficiency of *PinX1* has been found to result in telomere elongation and chromosome instability in MEF. Spontaneous HCC development has been observed in the *PinX1*$^{+/-}$ mice. The expression levels of PinX1 are similar when HCC and its surrounding non-tumor tissues in the *PinX1*$^{+/-}$ mice are compared[137]. These findings indicate that *PinX1* may function as a haploinsufficient TSG and is an essential factor for chromosome stability.

Shugoshin 1: Shugoshin 1 (SGO1) is a guardian of chromosome cohesion that is associated with the fidelity of chromosome segregation during mitosis[138]. In mice, heterozygous deficiency of *Sgo1* has been found to lead to persistent hepatocyte DNA damage, to promote azoxymethane (AOM)-induced HCC and to result in the development of spontaneous HCC in *Sgo*$^{+/-}$ mice. Interestingly, the SGO1 protein level has been shown to be higher in HCC tissue samples compared with the adjacent non-tumor tissues from the *Sgo1*$^{+/-}$ mice, suggesting that the wild-type allele of *Sgo1* is still present and is likely to be upregulated in HCC tissue[139]. In humans, upregulation of SGO1 expression has been observed in the HCC tissue samples compared with their adjacent non-tumor liver tissue samples[140]. These findings reveal that *Sgo1* helps maintain the accuracy of chromosome segregation and may function as a haploinsufficient TSG in HCC.

Genes related to various metabolic functions of the liver

The liver is an important metabolic organ and plays key roles in glucose, lipid, protein, bile acid and methionine metabolism. Dysregulation of metabolism may cause liver disease, including HCC[141]. Abnormal lipid metabolism, including decreased fatty acid oxidation, very low-density lipoprotein (VLDL) secretion or enhanced lipid uptake and lipogenesis, leads to hepatic steatosis, which is one of the risk factors for HCC. Lipid accumulation in hepatocytes results in lipotoxicity, oxidative stress, chronic liver damage and liver regeneration, which all contribute to hepatocarcinogenesis[142,143]. The effect of abnormal metabolic functioning, including lipid metabolism, methionine metabolism and bile acid metabolism, on hepatocarcinogenesis had been clearly demonstrated using mouse models.

The fatty acyl-CoA oxidase: The fatty acyl-CoA oxidase (AOX) protein, which is involved in lipid metabolism, is the first step enzyme of peroxisomal β-oxidation. AOX deficiency leads to steatohepatitis, oxidative stress, chronic liver damage and liver regeneration, as well as the development of spontaneous HCC in mice. Homozygous knockout of *AOX* results in the development of HCC due to sustained activation of peroxisome proliferator-activated receptor alpha (PPARα), indicating that abnormal lipid metabolism may contribute to HCC development[144].

BHMT and GNMT: Methionine metabolism is very important in maintaining the metabolic homeostasis of liver. One of the key metabolites is S-adenosylmethionine (AdoMet), which is the major methyl donor for methylation of several substrates, including DNA, RNA, histones, and various other small molecules. Abnormal methionine metabolism can cause fatty liver and HCC[145-147]. Reduced expression of the main enzymes involved in methionine metabolism, including methionine adenosyltransferase (MAT), glycine methyltransferase (GNMT) and betaine homocysteine (BHMT), have been observed in human HCC[148,149]. These findings suggest that impairment of methionine homeostasis may contribute to hepatocarcinogenesis.

BHMT is an enzyme that catalyzes the conversion of homocysteine (Hcy) to methionine. High levels of activity of BHMT have been detected in the livers of humans and mice. Disruption of *Bhmt* perturbs methionine metabolism and results in fatty liver and HCC development in mice. The fatty liver in *Bhmt*-deficient mice has been found to be due to a decrease in hepatic VLDL secretion[150]. These studies indicate that BHMT is important for methionine metabolism, the maintenance of liver homeostasis and the suppression of liver cancer.

GNMT is a methyltransferase that contributes to the maintenance of AdoMet homeostasis, which helps to avoid aberrant methylation. Loss of *Gnmt* causes fatty liver and HCC in mice. Aberrant methylation of DNA, which contributed to activation of the Janus kinase (JAK)/signal transducer of activators of transcription (STAT) pathway, has been observed in the livers of *Gnmt*-deficient mice[151]. These findings indicate that GNMT suppresses HCC development by maintaining AdoMet levels and allowing normal DNA methylation to occur.

Farnesoid X receptor and multidrug resistance 2: Bile acids are cholesterol metabolites and largely synthesized by hepatocytes. Hepatocellular bile acid synthesis and transport are highly regulated by canalicular transporter multidrug resistance 2 (MDR2) and nuclear transcriptional regulator farnesoid X receptor (FXR). Bile acids play key roles in the liver, including lipid metabolism, glucose metabolism and liver regeneration. Abnormal bile acid homeostasis may contribute to the development of fatty liver and HCC[152-154]. In mice, deficiency in the *Fxr* or *Mdr2* results in impairment of bile acids metabolism, an increase in inflammation, and the development of spontaneous HCC[155,156]. In humans, reduced expression levels of FXR and MDR2 have been observed in HCC tissue samples[157,158]. These findings suggest that deregulation of bile acid homeostasis may be involved

in hepatocarcinogenesis.

The Hippo signaling pathway

Aberrant cellular signaling pathways are critical events in the process of hepatocarcinogenesis. Previous studies have identified many critical signaling pathways involved in HCC development[159-161]. Previous studies in mice have revealed that dysregulation of many important signaling pathways contribute to the development of HCC; these included the Hippo pathway, the JAK/STAT pathway, the NF-κB pathway, the PI3K/AKT/mTOR pathway, the TGFβ pathway and the WNT/β-catenin pathway.

The Hippo signaling pathway is a highly conserved kinase cascade that regulates cell proliferation, cell survival and organ size control. The Hippo pathway can be divided into three major parts, namely the upstream regulators, the Hippo core kinase components, and the downstream target genes. The Hippo pathway is critical for the maintenance of hepatocyte quiescence and the regulation of liver size. Dysregulation of the Hippo pathway has been observed in many types of human cancers, including HCC[162-164]. In mouse models, the tumor suppressive role of various Hippo signaling components has been identified, including the core kinases MST1/MST2 and the upstream regulator NF1.

Mammalian sterile 20-like kinase 1 and 2: Mammalian sterile 20-like kinase 1 (Mst1) and Mst2 are the components of the Hippo core kinase cascade. The single knockout of *Mst1* or *Mst2* results in no obvious phenotypes in mice, which suggests the functional redundancy of Mst1 and Mst2 during embryonic development and hepatocarcinogenesis. However, hepatocyte-specific knockout of both the *Mst1* and *Mst2* genes causes an increase in hepatocyte proliferation, hepatomegaly and spontaneous HCC development in mice[165,166]. These studies demonstrate that Mst1 and Mst2 are required for organ size control and tumor suppression.

Neurofibromatosis type 2: Neurofibromatosis type 2 (NF2) is the positive regulator of the Hippo signaling pathway[167]. Nf2 deficiency leads to hepatic progenitor cell proliferation, hepatomegaly and hepatocarcinogenesis in mice. The tumor promoting phenotypes in the *Nf2*-deficient liver have been shown to be disrupted by loss of the Yes-associated protein (YAP), which is inhibited by the Hippo pathway[168,169]. These studies indicate that the tumor suppressor function of *Nf2* in HCC occurs *via* inhibiting YAP activation.

***JAK/STAT signaling pathway*:** JAK/STAT pathway is a plasma membrane to nucleus signaling pathway that regulates cell proliferation, differentiation and cell death. Activation of the JAK/STAT pathway is regulated by various positive (cytokine and tyrosine kinase) and negative regulators [protein tyrosine phosphatase (PTP) and suppressor of cytokine signaling (SOCS) proteins]. Constitutive activation of the JAK/STAT pathway is associated with tumorigenesis. The SOCS protein is one of the negative regulators of JAK/STAT pathway and its expression is decreased in many types of cancers including HCC[170-172]. The role of the negative regulators of the JAK/STAT pathway in hepatocarcinogenesis has been studied and evaluated using mouse models.

Suppressor of the cytokine signaling-1: Suppressor of the cytokine signaling-1 (SOCS1) is a negative regulator of JAK-mediated cytokine signaling *via* the protein's direct binding to JAK[173]. In humans, a lower level of SOCS1 expression has been observed in HCV-associated HCC samples compared with adjacent non-tumor liver tissue samples[174]. In mice, heterozygous deficiency of *Socs1* has been found to increase dimethylnitrosamine (DMN)-induced and diet-induced liver fibrosis; furthermore, heterozygous deficiency has also been found to promote DEN-induced hepatocarcinogenesis in the *Socs1*$^{+/-}$ mice. These results suggested that *SOCS1* is a haploinsufficient TSG in HCC.

Suppressor of the cytokine signaling-3: Suppressor of the cytokine signaling-3 (SOCS3) is a negative regulator for the JAK and interleukin (IL)-6-related signaling pathways[175]. In humans, expression of SOCS3 has been shown to be reduced in HCV-mediated HCC samples and this has been found to be accompanied by enhanced activation of STAT3. In mice, heterozygous deficiency of *Socs3* has been found to promote DEN-induced HCC in *Socs3*$^{+/-}$ mice[176]. These findings indicate that *SOCS3* may function as a haploinsufficient TSG in HCC.

Protein tyrosine phosphatase non-receptor type 11: Protein tyrosine phosphatase non-receptor type 11 (PTPN11) is a tyrosine phosphatase that dephosphorylates activated STAT3 and attenuates proinflammatory IL-6 signaling[177,178]. In humans, a decrease in PTPN11 protein expression has been observed in HCC tissue samples compared with the adjacent non-tumor tissue samples[179]. In mice, *Ptpn11* deficiency in hepatocytes has been shown to result in hepatic inflammation, cell death and hepatocellular adenoma formation. In addition, *Ptpn11* deficiency also promotes DEN-induced HCC development in mice. Furthermore, *Stat3* is required for the tumor promoting effect of *Ptpn11* deficiency to occur. Together, these studies indicate that *Ptpn11* functions as a tumor suppressor in HCC by controlling the oncogenic activity of *Stat3*.

Protein tyrosine phosphatase receptor type O: Protein tyrosine phosphatase receptor type O (PTPRO) is a receptor type of protein tyrosine phosphatase that

is frequently down-regulated in human HCC tissues through promoter hypermethylation[180,181]. Studies have revealed that *Ptpro* deficiency promotes DEN-induced HCC and enhances *Stat3* activity in mice[181,182]. These findings indicate that PTPRO suppresses HCC development *via* control of the activity of Stat3.

The nuclear factor-κB signaling pathway

Nuclear factor-κB (NF-κB) is an important transcriptional regulator of inflammation and is required for survival of hepatocytes in the liver. Upon tumor necrosis factor (TNF) stimulation, the IκB kinase (IKK) complex is recruited and activated by the kinase TAK1, and this then stimulates NF-κB activity by phosphorylating the negative inhibitor IκB protein[183]. Various studies have revealed that NF-κB is constitutively activated in many cancers including HCC. This suggests a tumor promoting role for NF-κB signaling[184]. However, evidence from different groups has indicated that NF-κB signaling may also have a tumor suppressor role in HCC. It is likely that NF-κB signaling has a dual role and that this role changes at different stages of carcinogenesis[185,186].

Cylindromatosis: Cylindromatosis (CYLD) is a deubiquitinase that inhibits the NF-κB pathway *via* deubiquitination of upstream regulatory factors[187]. In humans, reduced expression levels of CYLD have been observed in HCC tissue samples compared with the adjacent non-tumor tissue samples[188]. In mice, liver-specific disruption of CYLD triggers hepatic cell death, inflammation, fibrosis and spontaneous HCC development. Constitutive hyperactivation of TAK1, a NF-κB upstream regulatory factor, is required for liver pathogenesis in liver-specific CYLD knockout mice[189]. These findings show that CYLD suppresses hepatocarcinogenesis *via* control of the NF-κB signaling pathway.

Galectin-3: The galectin-3 is a down-stream target gene of NF-κB and plays an important role in inflammation response. Under normal conditions, galectin-3 is expressed in the bile duct epithelial and Kupffer cells, but not in the hepatocytes of livers[190,191]. However, expression of galectin-3 has been detected in human HCC cells, indicating that galectin-3 may be involved in HCC development[192]. In mice, homozygous knockout of galectin-3 results in fatty liver, inflammation, fibrosis and spontaneous HCC formation[193,194]. These studies indicate that galectin-3 plays an important role in hepatocarcinogenesis.

NF-κB-essential-modulator: NF-κB-essential-modulator (NEMO) is a regulatory subunit of the IKK complex and is essential for NF-κB activation. Ablation of NEMO blocks NF-κB activation in hepatocytes. In humans, loss of NEMO protein expression has been found in a substantial proportion of HCC tissue samples compared with their adjacent non-tumor tissue samples[195]. In mice, disruption of NEMO in hepatocytes leads to nonalcoholic steatohepatitis (NASH) and the spontaneous development of HCC[196]. These findings show that NEMO acts as a tumor suppressor in the liver.

TGF-β-activated kinase 1: TGF-β-activated kinase 1 (TAK1) is a kinase that activates the IKK complex upon TNF stimulation. Ablation of TAK1 in the hepatocytes of mice results in liver damage and the early onset of spontaneous HCC development, which suggests a tumor suppressor function for TAK1 in liver cancer[197].

PI3K/AKT/mTOR signaling pathway

The PI3K/AKT/mTOR pathway has a critical role in the regulation of cell growth, proliferation and metabolism. The PI3K/AKT/mTOR pathway is frequently activated in diverse cancers including HCC. The pathway is negatively regulated by phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and AMP-activated protein kinase (AMPK). Alterations in PTEN or the AMPK activator LKB1 have been observed in many types of human cancers[198,199]. Knock-out mice having loss of *PTEN* and *LKB1* have been generated and used to evaluate the roles of the PI3K/AKT/mTOR signaling pathway in HCC development.

Liver kinase B1: Liver kinase B1 (LKB1) is a serine/threonine kinase. LKB1 positively regulates downstream kinases including AMPK, which inhibits mTOR pathway. LKB1 has identified as the disease gene of Peutz-Jegher polyposis and cancer syndrome[200]. In addition, down-regulation of LBK1 has been observed in human HCC tissue samples compared with the adjacent non-tumor tissue samples[201]. In mice, heterozygous knockout of *Lkb1* leads to increased proliferation of hepatocytes and the development of spontaneous HCC. Loss of heterozygosity of the wild-type allele of *Lkb1* has been detected in the HCC tissues from the heterozygous knockout of *Lkb1* mice, indicating that *Lkb1* acts as a recessive TSG in HCC[202].

PTEN: PTEN is a phospholipid phosphatase that negatively regulates the highly oncogenic pro-survival PI3K/AKT signaling pathway[203]. In humans, expression levels of PTEN are decreased in HCC tissue samples compared with the adjacent non-tumor tissue samples[204]. In mice, homozygous knockout of *Pten* results in embryonic lethality. Furthermore, *Pten* heterozygous mice develop tumors in multiple organs including the liver. Loss of the wild-type *Pten* allele has been observed in liver tumors from $Pten^{+/-}$ mice[205-207]. Moreover, Pten deficiency in hepatocytes leads to steatohepatitis and HCC formation in mice[208]. These studies indicate that *Pten* functions as a recessive TSG in HCC.

Regulatory-associated protein of mTOR: Regulatory-associated protein of mTOR (RAPTOR) is one of the associated proteins of mTOR and is required for

mTOR activity. RAPTOR expression has been found to be up-regulated in advanced human HCC tissue samples compared with the adjacent non-tumor tissue samples, suggesting that the RAPTOR may contribute to hepatocarcinogenesis[209]. Hepatocyte-specific disruption of RAPTOR has been shown to result in hepatic damage, inflammation, fibrosis and the acceleration of DEN-induced hepatocarcinogenesis in mice. The HCC promoting effect of RAPTOR deficiency may be attributed partly to the hyper-activation of AKT in such livers[210]. These loss-of-function studies indicated that a persistent inhibition of mTOR may not be a suitable way to treat HCC.

TGF-β signaling pathway

TGF-β signaling is involved in many important pathways related to cell growth, including proliferation, motility and cell death. TGF-β inhibits hepatocyte proliferation in the liver, suggesting that it has potential tumor suppressive effects in HCC[211]. Although TGF-β has tumor suppressive functions in benign and early-stage tumors, it can also act as a tumor promoter in advanced-stage cancers. TGF-β has dual functions of tumor prevention and tumor promotion at difference cancer stages[212,213]. Scientists have applied genetically modified mouse models to evaluate the complex roles of the TGF-β signaling pathway in HCC development.

Embryonic liver fodrin: Embryonic liver fodrin (ELF) is an adaptor protein that mediates access to the receptor and SMAD3 activation in the TGF-β signaling pathway. In humans, ELF has been found to be significantly decreased in HCC tissue samples compared with the adjacent non-tumor tissue samples[214]. In mice, heterozygous deficiency of *ELF* leads to the development of fatty liver and spontaneous HCC, which is accompanied by enhanced activation of cyclin D1. These findings indicate that *ELF* functions as a haploinsufficient TSG in HCC.

Transforming growth factor β1: Transforming growth factor β1 (TGF-β1) is a negative factor that inhibits hepatocyte proliferation[215]. In mice, heterozygous deficiency of *TGF-β1* increases hepatocyte proliferation and promotes DEN-induced HCC in *TGF-β1*$^{+/-}$ mice. Tumors from *TGF-β1*$^{+/-}$ mice do not show loss of the wild-type allele of *TGF-β1*[216]. These findings indicate that the *TGF-β1* functions as a haploinsufficient TSG in HCC.

TGF-β type Ⅱ receptor: TGF-β type Ⅱ receptor (TβR-Ⅱ) is a receptor of TGF-β growth factor. In humans, down-regulation of TβR-Ⅱ has been frequently observed in HCC tissue[217]. In mice, heterozygous deficiency of *TβR-II* results in increased hepatocyte proliferation and promotes DEN-induced HCC carcinogenesis; in addition, a decrease of TβR-Ⅱ expression has been observed in the DEN-induced HCC tissue samples compared to the non-tumor liver in *TβR-II*$^{+/-}$ mice suggesting loss of the wild-type allele of *TβR-II* in HCC[218]. These studies show that *TβR-II* functions as a recessive TSG in HCC.

The WNT signaling pathway

The WNT/β-catenin signaling pathway plays critical roles in liver development, growth and metabolism. Moreover, WNT/β-catenin signaling is important for cancer development, including tumor initiation, growth and metastasis. Aberrant activation of WNT/β-catenin signaling has been observed in human HCC, suggesting that this pathway is involved in hepatocarcinogenesis[219-221].

Adenomatous polyposis coli: Adenomatous polyposis coli (APC) is the antagonist of WNT signaling and acts by enhancing β-catenin degradation. In humans, the expression level of the APC protein has been found to be decreased in HCC tissue samples compared to the adjacent non-tumor tissue samples[222]. In mice, liver-specific disruption of *Apc* causes activation of β-catenin signaling and spontaneous HCC formation[223]. These findings demonstrate that APC functions as a tumor suppressor in HCC *via* regulation of the β-catenin signaling.

MicroRNAs

MicroRNAs (miRNAs) are short noncoding RNAs and act as post-transcriptional regulators of gene expression. miRNAs may function as oncogenes or tumor suppressor genes through their targeting of various different genes involved in tumorigenesis. Deregulation of miRNA expression has been observed in human HCC indicating that miRNAs are involved in hepatocarcinogenesis. Importantly, the direct role of miRNA in hepatocarcinogenesis has been demonstrated using mouse models; examples of such miRNAs include miRNA-122 and miRNA-140[224-226].

miR-122 and miR-140: miR-122 is a major form of miRNA found in the liver. Downregulation of miR-122 has been observed in human HCC[227]. Disruption of miR-122 leads to hepatic steatosis, inflammation, fibrosis and spontaneous HCC in mice. The fatty liver in miR-122-deficient mice is caused by impaired VLDL secretion in the liver[228]. These studies demonstrate the metabolic and tumor suppressor function of miR-122 in livers. Another miRNA, miR-140, has also been suggested to act as a tumor suppressor in HCC. miR-140 deficiency results in enhanced NF-κB activity and promotes DEN-induced HCC development, indicating that the miR-140 functions as a tumor suppressor *via* regulation of the NF-κB activity in mice[229].

Miscellaneous

Nuclear receptor coactivator 5: Nuclear receptor coactivator 5 (NCOA5) is an estrogen receptor

coactivator that is able to enhance estrogen receptor α activity in the presence of estradiol[230]. In humans, expression of NCOA5 mRNA has been found to be decreased in about 40% of HCC tissue samples compared with their adjacent non-tumor tissue samples[231]. In mice, heterozygous deficiency of *Ncoa5* leads to fatty liver, inflammation, fibrosis and spontaneous HCC development. In addition, expression of the NCOA5 protein has been detected in HCC samples from *Ncoa5*$^{+/-}$ mice. These findings indicate that *Ncoa5* functions as a haploinsufficient TSG in HCC.

Regulatory subunit 1a of protein kinase A: Regulatory subunit 1a of protein kinase A (PRKAR1A) is the major component of type Ⅰ protein kinase A and regulates protein kinase activity[232]. Heterozygous deficiency of *Prkar1a* leads to multiple tumor formation events including HCC. Loss of heterozygosity of the *Prkar1a* allele was not observed in the tumors of *Prkar1a*$^{+/-}$ mice, suggesting that *Prkar1a* functions as a haploinsufficient tumor suppressor[233].

Nuclear receptor coactivator 2: Nuclear receptor coactivator 2 (NCOA2) is a transcriptional coactivator that regulates fasting hepatic glucose release by controlling the expression of glucose-6-phosphatase, which is the key enzyme of gluconeogenesis[234]. Homozygous deficiency of *Ncoa2* promotes DEN-induced hepatocarcinogenesis in mice revealing a tumor suppressor role for *Ncoa2* in liver cancer[235].

Nuclear factor erythroid 2-like 1: Nuclear factor erythroid 2-like 1 (NFE2L1; also known as NRF1, LCR-F1 and TCF11) is a transcription factor that controls the redox balance and lipid metabolism[236,237]. Hepatocyte-specific disruption of the *Nrf1* gene causes hepatic steatosis, cell death, inflammation, oxidative stress, fibrosis and spontaneous hepatic cancer in mice[238]. These findings suggested that *Nrf1* may function as a tumor suppressor in HCC by reducing oxidative stress in liver.

CONCLUSION

Spontaneous tumor formation largely occurs due to an accumulation of multiple somatic mutations. Discoveries from genetically modified mice are very informative and allow the identification of potential TSG based on clinical findings and characterization of carcinogenic mechanisms. HCC is tremendously heterogeneous at the pathological, clinical, genomic and molecular levels; this may be due to the natural function of liver, which serves as a major organ responsible for metabolism and detoxification of the whole body[239]. This situation also indicates the complexity of the signaling pathways active in the liver with multiple signaling pathways and cellular processes intercrossing in hepatocytes in order to regulate and control the cell cycle, metabolism and detoxification.

Inactivation of TSGs might involve a range of different modulations in addition to mutation, deletion and loss-of-heterozygosity; for example promoter silencing by DNA methylation. One of the therapeutic strategies is to take a TSG as a molecular target, for example *p53*. Not only the TSG itself can be a target; its interacting proteins and/or the downstream targets of the TSG are also candidates for suppressing tumor formation. In this regard, HCC mouse models, which recapitulate the pathology and progression of hepatocarcinogenesis in human HCC, provide a useful *in vivo* animal model for therapeutic testing and mechanistic studies under physiological conditions. Several TSGs are currently under evaluation in order to test whether they can serve as a target for treating lung or ovarian cancer[240]. Taking into consideration tissue specificity and the possibility of potential off-target problems, it may be useful to identify liver-specific TSGs for the development of precision medicine when tackling HCC.

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TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Targeting adeno-associated virus and adenoviral gene therapy for hepatocellular carcinoma

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Abstract

Human hepatocellular carcinoma (HCC) heavily endangers human heath worldwide. HCC is one of most frequent cancers in China because patients with liver disease, such as chronic hepatitis, have the highest cancer susceptibility. Traditional therapeutic approaches have limited efficacy in advanced liver cancer, and novel strategies are urgently needed to improve the limited treatment options for HCC. This review summarizes the basic knowledge, current advances, and future challenges and prospects of adeno-associated virus (AAV) and adenoviruses as vectors for gene therapy of HCC. This paper also reviews the clinical trials of gene therapy using adenovirus vectors, immunotherapy, toxicity and immunological barriers for AAV and adenoviruses, and proposes several alternative strategies to overcome the therapeutic barriers to using AAV and adenoviruses as vectors.

Key words: Hepatocellular carcinoma; Adeno-associated virus; Adenovirus; Virus vectors

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Core tip: This review summarizes the basic knowledge, current advances, and future challenges and prospects of adeno-associated virus (AAV) and adenoviruses as vectors for gene therapy of hepatocellular carcinoma. This paper also reviews the clinical trials of gene therapy using adenovirus vectors, immunotherapy, toxicity and immunological barriers for AAV and adenoviruses, and proposes several alternative strategies to overcome the therapeutic barriers to using AAV and adenoviruses as vectors.

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is the fifth leading cause of cancer death with an estimated > 33000 new cases per year in the United States, according to the American Cancer Society[1]. In China, HCC is also one of the five most common cancers, whose incidence rate ranks fourth and mortality ranks second, with 18.43 per 10000 among all types of cancers[2,3]. Surgery, chemotherapy, and radiotherapy are still the most common therapeutic options for HCC. Surgery is the first choice for early stage disease and offers a cure rate of 10%-20% in HCC patients. However, liver cancer recurrence rate remains high after tumor resection because of the aggressive traits of HCC, such as metastasis and chemo- or radiotherapy resistance[4,5]. Although tremendous efforts have been made to improve the anti-cancer effect for HCC, there are still no ideal therapeutic strategies. Gene therapy has attracted much interest as a novel promising therapeutic method since the first approved successful clinical trial for children with severe combined immunodeficiency (SCID) in 1990[6]. Henceforth, gene therapy has rapidly developed as a strategy for single gene hereditary diseases, infections, and cancer, using various viral or nonviral vectors[7,8]. Currently, most effective vectors are derived from recombinant viruses including adenovirus, adeno-associated virus (AAV), vaccinia virus, retrovirus, lentivirus, herpes simplex virus, Epstein-Barr virus, and chimeric viruses[8,9]. Since we proposed the Cancer Targeting Gene Virotherapy (CTGVT) strategy by combining cancer gene therapy and virotherapy using oncolytic adenoviral vector in 2001, numerous studies have been performed in HCC and other cancers using gene therapy[10-12]. Additionally, due to its distinct merits, AAV is considered as the prime candidate vector for clinical gene therapy for various liver diseases[13,14]. This review describes the advances, therapeutic mechanisms, and current challenges in using AAV and adenoviruses as vectors for gene therapy of HCC.

BIOLOGY OF AAV AND ITS APPLICATION AS A VECTOR FOR HCC

AAV, a member of the parvovirus family, has a single-stranded DNA genome of approximately 4.7 kb. The genome consists of two open reading frames (ORFs) rep and cap driven by three promoters (P5, P19 and P40), which are flanked by the 145 nucleotide long inverted terminal repeat sequences. The rep encodes four overlapping functional proteins (Rep78, Rep68, Rep52, and Rep40), which play a part in viral replication, transcriptional control, and accumulation of single-stranded progeny genomes, and the cap contains three capsid proteins (VP1, VP2, and VP3) functioning in the generation of infectious particles[15].

Essential properties of AAV vector

Since Hermonat *et al*[16] first used AAV as a vector for transgene delivery into cultured mammalian cells, AAV-based vectors have undergone rapid development in recent decades. Unlike the wild-type AAV that is able to integrate into the host genome, exogenous genes delivered by recombinant AAV vector can be persistently expressed in an episomal state[17,18]. AAV vectors have some others advantages, including a broad host spectrum that allows them to infect both non-dividing and dividing cells and low pathogenicity or no cytotoxicity[19].

Presently, there are more than 11 different serotypes of AAVs and over 100 new AAV variants to be identified and engineered into vectors. AAV2 serotype was the first to be used for the transfer of transgenes into host cells and has emerged as a promising carrier for clinical gene transfer in several single genetic disorders[20]. As the first clinical gene therapy medicine (AAV2-LPL) authorized by European Medicines Agency (EMA)[21], it largely promotes the development of AAV vectors for clinical transgene therapy. Besides AAV2, AAV1 and 3-9 serotypes were engineered as gene transfer vectors. AAV1 vector has a higher transfer rate in muscle than AAV2[22]. AAV1, 4, and 5 can transfer genes efficiently into muscles, lung, and central nervous system[8,23]. The serology of AAV6 is almost identical to that of AAV1, and the AAV6 vector was designed to transfer primary human hematopoietic stem cells into a mouse model[24]. AAV7 and AAV8 are two new members of the AAV family isolated from rhesus monkeys[25]. Furthermore, the efficiency of gene transfer to liver with AAV7 and 8 vectors was higher than that achieved using AAV2, although a variety of host factors may influence this important parameter, such as pre-existing antibodies, gender, and transgene immunity[25-27]. So far, the serological profiles of AAV10 and AAV11 are not well characterized[28,29]. AAV12 is a novel AAV serotype with transduction activity independent of sialic acid and heparan sulfate proteoglycan[30]. The AAV variants are not further identified and exploited as vectors due to the lack of serological profiling.

AAV vector development for HCC

In recent years, many important breakthroughs have been achieved in gene therapy in several disease types, including genetic diseases and cancer. Up to January 2015, there were 127 trials of gene therapy mediated by AAV vectors registered at the Journal of Gene Medicine Clinical Trial website[31]. These trials have been performed for the treatment of various diseases, including cancer and monogenic, neurological, ocular,

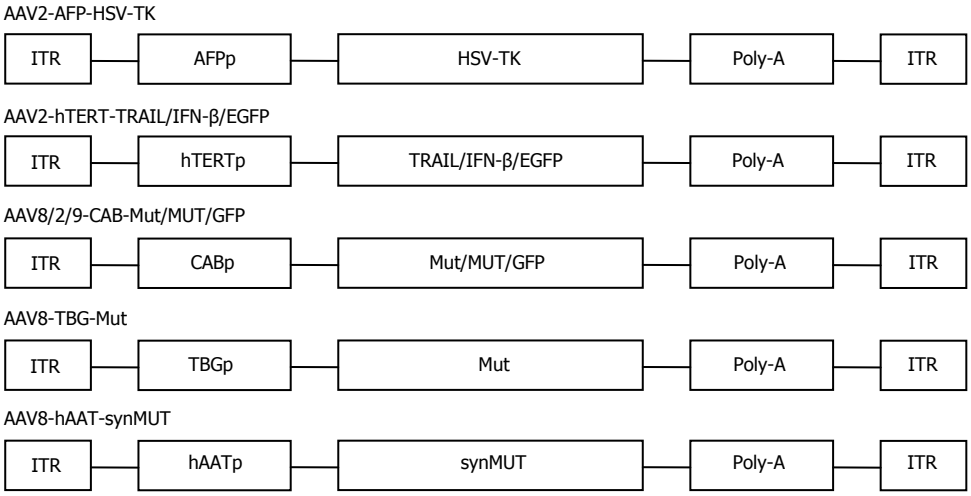

Figure 1 Schematic of adeno-associated virus vectors packaged into adeno-associated virus serotypes 2, 8, and 9 with different promoters used for gene delivery. AFPp: Human α-fetoprotein promoter; hTERTp: A truncated human telomerase; CBA: Chicken β-actin; TBG: The liver-specific thyroxine-binding globulin; hAAT: Human α1-antitrypsin.

cardiovascular, and infectious diseases. The landmark of AAV-mediated clinical studies is the development of inherited retinal diseases and anerythrochloropsia gene therapy. AAV-mediated PRE65 gene expression efficiently recovered visual function in patients with Leber's congenital amaurosis with controlled safety and efficacy of gene transfer[32-34]. These encouraging results widened the clinical applications of AAV vectors, including gene therapy of cancer.

AAV vectors are engineered for delivery to patients suffering from liver diseases, including familial hypercholesterolemia, viral hepatitis, and hepatic malignancies. The first gene therapy experiment for HCC using AAV vectors was carried out by Su *et al*[35]. The constructed recombinant AAV virus, which carried the herpes simplex virus thymidine kinase (TK) gene driven by the human α-fetoprotein (AFP) enhancer and the albumin promoter, resulted in a selective killing effect on AFP-positive HCC cells but not non-hepatocyte tumor cells or AFP and albumin-negative hepatic tumor cells[35]. Moreover, the dose required to kill the cancer cells was inversely proportional to the level of AFP expression in the cells.

Actually, besides suicide-gene-directed enzyme/pro-drug therapy, gene therapy using AAV covers numerous therapeutic methods, such as inhibition of oncogenes and re-expression of tumor suppressor genes, immunotherapy, anti-angiogenesis therapy, and combination therapy[19]. There are a variety of targeting strategies for AAV application, including transcription-targeted, receptor-targeted, and conjugate-targeted strategies and various alternative AAV serotypes[8]. The application of human telomerase reverse transcriptase (hTERT) promoter was a good candidate for transcription-targeted cancer gene therapy[13]. In general, cancer cells have high telomerase activity, and hTERT, a catalytic subunit of the telomerase, is transcriptionally upregulated exclusively in about 90% of cancer cells[36]. Thus, telomerase is an excellent tumor marker. We first constructed a novel AAV vector targeting telomerase activity and investigated its targeting capability and anti-tumor potential though carrying a human interferon-β gene[13]. The recombinant virus AAV-hTERT-human interferon (hIFN)-β displayed cancer-specific hIFNβ expression and cytotoxicity in various human cancer cells *in vitro* and suppressed tumor growth in nude mice of lung cancer A549 and colorectal cancer SW620 xenografts[7]. In addition, we and others generated a recombinant AAV vector containing the tumor necrosis factor alpha related apoptosis inducing ligand (TRAIL) gene under the control of the hTERT promoter. The AAV-hTERT-TRAIL virus exhibited cancer-specific cytotoxicity and apoptosis that which significantly suppressed the growth of HCC xenograft tumors[37,38]. These results indicated that AAVs in combination with hTERT-mediated therapeutic gene expression provide a promising targeting approach for developing effective therapy for HCC (Figure 1).

AAV-mediated gene therapy of HCC has made great progress in other research areas. Intraportal injection of recombinant AAV carrying kallistatin gene suppressed hepatic and subcutaneous HCC tumors through antiangiogenic and antiproliferative activities[39]. Ling *et al*[40,41] validated that AAV serotype 3 is an excellent vector in efficiently transducing human liver cancer because AAV3 uses human hepatocyte growth factor receptor as a cellular co-receptor for binding and entry into these cells, which implies that AAV3 vectors can be applied to gene therapy of liver cancer. In addition, AAV8 may be the best liver-specific transfer vector and has good prospects for liver cancer gene therapy[42]. In particular, RNA interference (RNAi)-based approaches, such as antisense hypoxia-inducible factor-1α and microRNA (miRNA)-targeted therapy mediated by AAV have been recently ex-

ploited as new anti-cancer treatments for HCC[43-45]. Systemic administration of AAV-mediated miR-26a delivery efficiently inhibited HCC cell proliferation, induced tumor-specific apoptosis, and suppressed tumorigenesis in a murine liver cancer model[45]. Other than the general strategy for miRNA replacement therapies, inhibiting the oncogenic miR-221 by miRNA sponge was developed for therapy of HCC, in which AAVs were genetically modified to drive the expression of multiple binding sites for miR-221[44].

Combination therapy is an important tactic for clinical cancer therapy. AAV-mediated gene therapy is widely considered as a potential adjuvant of other therapies. We attempted combination therapy with AAV-hTERT-TRAIL and cisplatin, which could have a synergistic therapeutic effect on HCC. As expected, treatment with both AAV-hTERT-TRAIL and cisplatin exhibited a stronger inhibitory effect and induced more significant apoptosis compared with either agent alone in HCC cells and animal tumors[46]. Other studies showed that radiotherapy can enhance transduction of HCC cells by recombinant AAV *in vitro* and *in vivo*[47]. Even the cocktail viral gene therapy, combining the effect of AAV transducing hepatocyte growth factor dringle 1 and adenovirus transducing p53, significantly induced tumor cell death, inhibited tumor angiogenesis and tumor growth, and prevented tumor metastasis in HCC models[48]. Multigene-based combination therapy is an effective practice in cancer gene therapy. AAV-mediated coexpression of apoptin and interleukin (IL)-24 in HCC significantly suppressed the growth and induced apoptosis of HepG2 cells *in vitro* and in xenograft nude mice[49]. An AAV6 serotype designed for dendritic-cell-based cancer immunotherapy can be a useful targeting approach for efficient HCC therapy[50], which could lay the foundation for further development for AAV-mediated HCC immunotherapy.

THERAPEUTIC APPLICATION OF ADENOVIRUS IN HCC

Adenovirus has been the most common gene transfer vector for cancer gene therapy in past decades because of its unique advantages, such as broad tropism for infecting many human tissues including hepatocytes, capability of transducing nonreplicating cells and replicating cells, and easy acquisition of high titers, benefiting clinical use and efficient transgene expression[51,52]. Numerous studies have reported the potential application of adenovirus-mediated gene therapy for a wide variety of diseases and indicated their beneficial effects, tolerability, and safety. The following three aspects describe oncolytic adenovirus vector, adenovirus-mediated immune treatment, and clinical trials for HCC therapy, respectively.

Oncolytic adenovirus vector

Currently, oncolytic viruses represent a group of promising anti-cancer agents with the ability to lyse infected cancer cells but not normal cells. The oncolytic viruses include genetically modified adenovirus, vaccinia virus, herpes simplex virus, and reovirus[10]. ONYX-015, an oncolytic adenovirus designed with the E1B 55-kDa gene deletion, was engineered to replicate in and lyse cancer cells selectively[53]. Clinical trials using ONYX-015 alone or in combination with chemotherapy have been widely performed in patients with head and neck cancer, which achieved an obvious anti-cancer effect[54,55]. In addition, many targeted strategies based on oncolytic adenoviruses were conceived, and they exhibited potent anti-tumor activity in various preclinical studies[11,56,57]. Previously, our CTGVT, which combined the superiority of gene therapy and virotherapy, brought new hope for cancer therapy[10,58]. Similar to ONYX-015, the novel oncolytic adenovirus ZD55 was first engineered with E1B55-kD protein deletion based on CTGVT, and was combined with anti-cancer gene therapy to form the ZD55-gene system[58]. Our many experiments confirmed that the ZD55 gene exerted a potent anti-tumor effect in multiple tumor cell lines and mouse models through the synergetic mechanisms of the oncolytic action of the virus itself and overexpression of anti-tumor genes[11,52,59-63]. In particular, the novel oncolytic adenovirus ZD55-Smac increases anti-tumor activity of ZD55-TRAIL against HCC *via* a synergetic apoptotic effect[64]. In general, HCC frequently displays a high resistance to TRAIL-mediated cell death due to high expression of inhibitor of apoptosis proteins (IAPs), while Smac strongly inhibits IAPs and increases sensitivity of HCC cells to TRAIL[65]. Thus, combination treatment with ZD55-Smac and ZD55-TRAIL led to rapid and potent activation of apoptosis in HCC cells and eliminated completely tumor xenograft in all treated animals, which could provide a useful strategy for therapy of HCC[64].

Furthermore, numerous other combination strategies were adopted in HCC therapy based on the oncolytic adenovirus ZD55 system. Chu *et al*[62] found that adenoviral vector expressing cylindromatosis (CYLD) augments anti-tumor activity of ZD55-TRAIL by suppression of nuclear factor-κB survival signaling in HCC. Pan *et al*[66] constructed ZD55-mediated X-linked inhibitor of apoptosis protein (XIAP)-shRNA by RNAi to inhibit high XIAP of HCC cells and concluded that combination of ZD55-shXIAP and ZD55-TRAIL led to synergistic anti-tumor activity in experimental HCC. Moreover, the combination of XAF1, IL-24, or Smac-armed ZD55 and chemotherapy exhibited significantly enhanced suppression of HCC growth by a synergistic mechanism, especially in the induction of apoptosis[67-69].

However, traditional oncolytic adenovirus lacks the ability to target liver cancer. To improve the anti-cancer effect of oncolytic adenovirus in liver cancer, three main strategies have been developed in recent years. First, the transcription-targeted strategy is designed

Table 1 Liver- or hepatocellular carcinoma - specific promoters in adeno-associated virus vectors

Promoter	Target	Delivery gene	Ref.
CBA	Liver	Reporter, *Flk-1, Mut, MUT, GFP*	[15,87-89]
TBG	Liver	Canine factor *IX, IDS. Mut*	[15,90,91]
hEF1α	Liver	*IFN-α*	[92]
hAAT	Liver	*hFIX, synMUT*	[15,93]
Mouse albumin	Liver	*HGAA, tTA, EGFP*	[94]
Albumin + α fetoprotein	Hepatocellular carcinoma	*HSV-TK*	[35,95]
hTERT	Hepatocellular carcinoma	*TRAIL*	[8]
survivin	Hepatocellular carcinoma	*TSLC1*	[70]

CBA: Chicken β-actin; TBG: The liver-specific thyroxine-binding globulin; hAAT: Human α1-antitrypsin.

using cancer- or tissue-specific promoter to control the expression of viral genes essential for replication[70,71], which results in selective expression of viral genes, propagation of virus progeny, and tumoricidal activity[9,10]. We designed AFP-regulated oncolytic adenovirus AD and obtained a better anti-cancer effect in HCC than other types of cancer[72-76]. Golgi protein (GOLPH2) is an excellent HCC marker[77,78], therefore, we constructed a novel GOLPH2-regulated oncolytic adenovirus GD55 that targets HCC[79]. We showed that the novel GD55 had higher adenovirus replication ability and infectivity in liver cancer cells than the common oncolytic adenovirus ZD55. In addition, GD55 exerted a significant growth-suppressing effect on HCC cells or xenograft but caused little damage to normal liver cells, which may provide a promising oncolytic virus for future liver cancer treatment[79]. The second strategy targets the tumor signaling pathway through deletion or mutation of key adenovirus genes or some bases that are necessary for adenovirus replication in normal cells but not in tumor cells. The classical design of oncolytic adenovirus is ONYX-015 and ZD55, whose E1B 55 kDa gene is deleted by genetic engineering[53,58]. They are supposed to target and lyse p53-dysfunctinal tumor cells preferentially but not adjacent normal cells[80], but further study demonstrated that the adenovirus mutant enhanced the viral mRNA late nuclear transport and oncolysis[81]. We also noted inhibition of liposarcoma by histone deacetylase inhibitor occurs irrespective of p53 mutational status but *via* targeting of the MDM2-p53 signaling axis and phosphatase and tensin homolog[82]. Another modification was a 24 base pair deletion in the E1A region, such as oncolytic virus Ad5-E1A$_{(\Delta 24)}$; which is responsible for binding retinoblastoma (Rb) protein, and its replication is restricted in Rb-inactive arrested cells and exhibits tumor-selective capability[56,71,83]. The third approach is the receptor-targeted or capsid-modified strategy. Adenovirus can efficiently infect host cells by binding to specific receptors on the target cell surface with fibers on the capsid. Thus, modification of the adenovirus capsid may improve the binding ability of adenovirus to target cells. The adenovirus vector with genetically modified fibers (RGD-4C or chimera fibers of different serum types) demonstrated expanded tropism *via* utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism[84,85]. Otherwise, efficient and selective gene transfer into primary human tumors using single-chain antibody-targeted adenoviral vectors with native tropism abolished the specific targeting ability[86]. In Table 1, we sum up Liver- or hepatocellular carcinoma- specific promoters and delivery gene in adeno-associated virus vectors for the past few years.

Adenovirus-mediated immunotherapy

The three traditional anti-cancer therapies (surgery, radiation, and chemotherapy) often carry risks and/or cause adverse side effects and show limited efficacy, particularly for late-stage cancer. The fourth option is cancer biotherapy, including oncolytic viruses and immunotherapy, which has emerged as a promising therapy in preclinical trials and cancer patients. Immunotherapy was considered the Breakthrough of the Year for 2013 and 2014 because of the efficacy of antibodies against cytotoxic T-lymphocyte-associated protein (CTLA)-4, programmed death (PD)-1, ligand 1, and chimeric antigen receptor[96,97]. Encouragingly, CD19-targeted T cells achieved complete remission in children and adults with chemotherapy-refractory acute lymphoblastic leukemia[98]. These also brought inspiration and confidence to acquire the expected therapy effect either *via* oncolysis or antitumor immunity by recombinant oncolytic adenovirus.

Fortunately, people began to realize that oncolytic adenoviruses cause the immune system to stimulate an anti-tumor immune response[99]. A novel oncolytic adenovirus, Ad5D24-CpG, was engineered by inserting 18 immunostimulatory islands into Ad5D24. This virus showed increased anti-tumor activity *via* the stimulation of Toll-like receptor 9 and inactivation of myeloid-derived suppressor cells in modified virus-treated mice[100]. Moreover, to achieve superior anti-cancer immunity, oncolytic adenoviruses are often designed to express immunostimulatory molecules including CD40L, IL-2, IL-12, IL-24, and granulocyte-macrophage colony-stimulating factor (GM-CSF)[101]. Among these constructs, oncolytic adenovirus coding for GM-CSF (Ad5-D24-GMCSF) was a typical agent, and it induced anti-tumor immunity in cancer patients with advanced solid tumors refractory to standard therapies, indicating that the treatment was safe. The

Table 2 Overview of clinical trials that use adenovirus vector for hepatocellular carcinoma therapy

Adenovirus	Phase	Trial	Status	Route	Notes
ADV-TK	Ⅱ	NCT00300521	Completed	it	As a single agent
TK99UN	Ⅰ	NCT00844623	Completed	it	As a single agent
AdVhAFP	Ⅰ/Ⅱ	NCT00669136	Terminated	im	Combined with AFP and GM-CSF
AdVhAFP	Ⅱ/ⅢA/ⅢB/ⅣA	NCT00093548	Withdrawn	im and id	Combined with AFP and GM-CSF
ADV-TK	Ⅱ	NCT02202564	Completed	Intrahepatic	Double-dose
Ad5CMV-p53	Ⅰ	NCT00003147	Terminated	Percutaneous injection	As a single agent
Adenovirus Type 5	Ⅲ	NCT01869088	Recruiting	Arterial infusion	Combined with TACE
rAd-p53	Ⅱ	NCT02418988	Recruiting	Arterial injection	Combined with TACE

TACE: Transcatheter arterial chemoembolization; ADV-TK: Adenovirus thymidine kinase; AdVhAFP: α-fetoprotein adenoviral vector; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TK99UN: Adenoviral vector containing thymidine kinase of herpes simplex virus; im: Intramuscularly; it: Intratumoral; id: Intradermally.

tumor completely disappeared in 2/20 patients[102].

Further modifications were made in the following approaches to improve clinical anti-tumor immunological benefit. The adenovirus serotype 5 capsid was replaced with serotype 3 fiber knob to form chimeric adenovirus vector Ad5/3-D24-GMCSF, avoiding the problem of coxsackie-adenovirus receptor downregulation in advanced tumors[99]. Another immunostimulatory molecule of interest is CD40L, a multifunctional protein. Oncolytic adenovirus encoding CD40L led to a strong anti-tumor effect[103]. Efficient targeted cancer immunotherapy was achieved with oncolytic adenovirus coding for a fully human monoclonal antibody specific for CTLA-4 (Ad5/3-Δ24aCTLA4), avoiding the severe immune-related adverse events by systemic administration of monoclonal antibodies ipilimumab or tremelimumab blocking CTLA-4[104]. It also suggests the feasibility of immunotherapy with oncolytic adenovirus-mediated CTLA4 antibody[105].

An alternative means of anti-tumor immunotherapy is oncolytic adenovirus-vector vaccines. Orally delivered oncolytic adenovirus vaccines have been utilized to prevent adenovirus-induced respiratory illness in military recruits, demonstrating safety and high efficacy[106]. The experience suggested that oral administration of live oncolytic adenoviruses holds promise for immunization against liver cancer and other infectious diseases because live adenoviruses can express intact tumor-associated antigens as transgenes in infected cells[107]. Another novel approach is incorporation of antigenic epitopes into adenovirus capsids, eliciting the strongest humoral and cell-mediated immune responses, both prior to and during virus replication, against cancer and infection[106,108,109].

Clinical trials for adenovirus-associated HCC therapy

The first gene transfer with recombinant replication-defective adenovirus was successfully implemented in HCC cells in 1995, which led to induction of sensitivity to ganciclovir in human HCC cells by adenovirus-mediated herpes simplex virus TK gene[110]. Since then, 293 papers have been published in the field of adenoviruses and HCC, and 317 gene therapy studies using adenovirus vectors have been registered at ClinicalTrials.gov. Adenoviruses are the most frequently used gene transfer vectors in clinical trials according to the Journal of Gene Medicine Clinical Trial site[31]. However, at the time of the preparation of this review (May 2015), official sources listed only eight clinical trials that described the status (Table 2), efficacy, and safety of adenovirus-associated therapy in HCC.

In particular, there are two studies that are currently recruiting participants. Recombinant human adenovirus type 5, with an E1B gene deletion, combining transcatheter arterial chemoembolization (TACE), is being tested as a stand-alone therapeutic intervention in a phase Ⅲ trial in patients with advanced HCC not amenable to surgery or local ablative therapy (NCT01869088). A phase Ⅱ trial of rAd-p53 artery injection combined with TACE in adults with HCC is being sponsored by Shenzhen Sibiono Genetech (NCT02418988). rAd-p53 was the first approved adenovirus agent worldwide for the treatment of head and neck cancer, and great success was achieved in these patients, especially when combined with chemotherapy or radiotherapy. In addition, three clinical trials have recently been completed using adenovirus vectors. The preliminary safety and efficacy of double-dose adenovirus-mediated adjuvant therapy (Adv-TK, adenovirus expressing TK) was evaluated in phase Ⅱ trials, resulting in improved outcome of liver transplantation in patients with advanced HCC (NCT02202564). In addition, liver transplantation with Adv-TK gene therapy improved survival in patients with advanced HCC (NCT00300521). Intratumoral injection of TK99UN (an adenoviral vector containing TK) was assessed in a phase Ⅰ clinical trial in adult HCC patients (NCT00844623). Two trials using adenovirus vectors were terminated for undisclosed reasons. A phase Ⅰ trial of the effectiveness of gene therapy with Ad5CMV-p53 was also terminated in patients with liver cancer that could not be surgically removed (NCT00003147). A suspended phase Ⅰ/Ⅱ trial is testing immunization, safety, and toxicity of AFP plus GM-CSF plasmid prime and AFP-armed adenoviral vector (Adv-hAFP) in patients with locoregionally pretreated HCC (NCT00669136). A vaccine therapy study using Adv-hAFP was halted prior to enrollment

for treating patients with stage Ⅱ, ⅢA, ⅢB, or ⅣA liver cancer (NCT00093548).

EXISTING PROBLEMS AND CHALLENGE

Toxicity and immunological barriers for AAV

The nonpathogenic feature of AAV endows it as a promising gene therapy vector with little or no acute toxicity to the host. Results from gene therapy trials with AAV vectors, especially some exciting results from clinical studies of hemophilia B, congenital blindness, and familial lipoprotein lipase deficiency, have confirmed their therapeutic potential[110,111].

However, some of the limitations of *in vivo* AAV gene transfer have emerged. First, the host immune response to AAV capsid is an important obstacle to safety and efficacy of AAV-vector-mediated gene transfer *in vivo*[112,113]. AAV2-capsid-specific cytotoxic T cells were detectable following AAV2-mediated hepatic delivery of factor IX in hemophilia B patients, which resulted in killing and clearance of transduced hepatocytes and affected the therapeutic efficacy. It was hypothesized that this was caused by rejection of transduced hepatocytes by AAV-capsid-specific memory $CD8^+$ T cells reactivated by AAV vectors, because these patients harbor a population of capsid-specific memory cytotoxic T cells formed during childhood infection with wild-type AAV2[114]. Second, the humoral immune response is a universal obstacle in virus-mediated gene transfer *in vivo*, largely affecting the therapeutic efficacy. Conceivably, both the transgene protein and AAV capsid can produce relevant antibodies. Anti-AAV capsid neutralizing antibodies are highly prevalent and detectable in two-thirds of the population. Even high titer AAV neutralizing antibodies can completely inhibit vector transduction to the target tissue, leading to lack of efficacy[113]. Although the integration potential of AAV into the host genome offers long-term transgene expression in animal experiments or clinical trials, there is a risk of insertional mutagenesis that may induce carcinogenesis[115]. In particular, a high incidence of HCC was observed in mice or other mouse models after systemic delivery of AAV gene therapy vector[115]. The hepatic genotoxicity may be caused by AAV integration into the RNA imprinted and accumulated in the nucleus (Rian) locus, resulting in overexpression of proximal miRNAs and retrotransposon-like 1 associated with HCC[15].

Problematic limitations for oncolytic adenovirus

Adenovirus has a broad range of vertebrate hosts, including humans, and commonly causes illnesses such as mild respiratory infections and cold-like syndrome in young children. Although wild-type adenovirus can kill some cancer cells, it also has many side effects. Numerous modified oncolytic viral constructs, such as H101, Ad-p53, and Ad5-D24-GMCSF, have indicated potent anti-tumor efficacy in patients with solid cancers refractory to standard therapeutics with limited or no toxicity to normal tissue[9]. However, there currently are some inevitable obstacles for clinical application of systemic adenovirus-mediated gene therapy. These are high prevalence of neutralizing antibodies, induction of immune and inflammatory responses, high promiscuity due to widespread expression of the coxsackie-adenovirus receptor, and adenovirus sequestration by the liver[116]. The approximately 36 kb genome of adenovirus comprises a variety of structural, replication, and regulatory genes, resulting in the complexity and uncertainty of the toxic effect induced by oncolytic adenovirus during systemic administration. The representative oncolytic adenovirus mutant is ONYX-015 (designed by Onyx Pharmaceuticals). Although phase Ⅰ and Ⅱ clinical trials of ONYX-015 were completed in patients with various solid tumors, a phase Ⅲ trial for the treatment of recurrent head and neck cancer patients was suddenly repealed because of possible safety problems a decade ago. Therefore, improving anti-cancer efficacy and reducing toxic effects and immune response to adenovirus vectors remain potential challenges to successful HCC therapy.

FUTURE PROSPECTS

With the rapid development of HCC incidence and mortality in China, there is an urgent need for innovative, alternative therapies for HCC patients. Despite anti-tumor efficacy being achieved by AAV- or adenovirus-mediated gene therapy in experimental liver cancer models, researchers soon realized its limitations and ongoing efforts are being made to resolve these limitations. Efficient transfer of genes/small RNAs to the majority of cancer cells is still unrealistic for solid tumors[117]. To date, there are 17 trials using AAV vectors and 154 trials using adenoviruses for gene therapy of cancer registered at the Wiley Clinical Trial site[31], although none of these trials for AAV and only eight for adenoviruses are investigating liver cancer. Therefore, more efficient AAV or adenovirus vectors targeting HCC should be designed to achieve successful treatment of liver cancer. Current strategies are mainly aimed at chemical modification of the virus capsid, serotype substitution of different virus types, and hybrid vectors combining viral and synthetic vectors to improve therapeutic efficacy for HCC. These new strategies have gradually demonstrated that the modified vectors have the ability to escape neutralizing antivirus antibodies, to overcome liver tropism, and to reduce humoral and cellular immune responses and liver toxicity even after systemic virus administration, while maintaining their natural biological activity[118]. In addition, the various combination strategies between different virus vectors or gene therapy and conventional/cell therapy can optimize the efficacy of AAV or adenovirus-mediated therapy. Thus, we believe that optimal scheduled

combinatorial regimens will likely have promising antineoplastic effects in the field of gene therapy with modified virus vectors.

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REVIEW

Xenobiotics and loss of tolerance in primary biliary cholangitis

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Abstract

Data from genome wide association studies and geoepidemiological studies established that a combination of genetic predisposition and environmental stimulation is required for the loss of tolerance in primary biliary cholangitis (PBC). The serologic hallmark of PBC are the presence of high titer anti-mitochondrial autoantibodies (AMA) that recognize the lipoyl domain of the mitochondrial pyruvate dehydrogenase E2 (PDC-E2) subunit. Extensive efforts have been directed to investigate the molecular basis of AMA. Recently, experimental data has pointed to the thesis that the breaking of tolerance to PDC-E2 is a pivotal event in the initial etiology of PBC, including environmental xenobiotics including those commonly found in cosmetics and food additives, suggesting that chemical modification of the PDC-E2 epitope may render its vulnerable to become a neo-antigen and trigger an immune response in genetically susceptible hosts. Here, we will discuss the natural history, genetics and immunobiology of PBC and structural constraints of PDC-E2 in AMA recognition which makes it vulnerable to chemical modification.

Key words: Antimitochondrial autoantibodies; Primary biliary cholangitis; Pyruvate dehydrogenase E2; Breaking of tolerance; Xenobiotics

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Core tip: Environment influences immune functions. In this paper, we examine how environmental chemicals can trigger autoimmunity in an organ specific autoimmune disease, primary biliary cholangitis (PBC). PBC is liver specific autoimmune disease characterized by high titer of anti-mitochondrial autoantibodies directed against the E2 subunit of pyruvate dehydrogenase (PDC-E2) lipoyl domain. Here,

we present experimental evidence from quantitative structure-activity relationship and animal models that xenobiotic modification of the PDC-E2 lipoyl domain could lead to loss of self-tolerance and is a pivotal event in the initial etiology of PBC in genetically susceptible hosts.

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INTRODUCTION

The loss of tolerance is a central theme in autoimmunity and genetics and geoepidemiological studies have reflected that environmental factors contribute to this breach of tolerance[1-10]. This thesis is exemplified in primary biliary cholangitis (PBC), a prototype organ specific autoimmune disease[11]. The mechanism of how immunological tolerance is broken in PBC is still enigmatic[12]. Importantly, the autoantigen recognized by AMA was first cloned in 1987 and subsequently identified as the E2 subunit of pyruvate dehydrogenase (PDC-E2)[13,14]. The epitopes of AMA have been mapped to the highly conserved lipoic acid binding domain of the 2-oxo acid dehydrogenases including PDC-E2, branched chain 2-oxo-acid dehydrogenases (BCOADC-E2), oxoglutarate dehydrogenase (OGDC-E2) and the E3 binding protein (E3BP)[13-16]. Extensive efforts in defining the target mitochondrial autoantigens, T and B cell epitopes, the innate and adaptive immune responses, the immunobiology of the biliary epithelium, and the pathology of biliary duct epithelial cell destruction have greatly advanced our knowledge of the molecular mechanisms in tissue damage[13,17-29]. This focus of this review is to provide a comprehensive view of our current understanding on the natural history, genetics and immunobiology of PBC with emphasis on experimental data that illustrate the loss of tolerance to PDC-E2 is a pivotal event in the etiology of PBC[25,30-32].

NATURAL HISTORY AND GENETICS OF PRIMARY BILIARY CHOLANGITIS

Primary biliary cholangitis (PBC), previously known as primary biliary cirrhosis[33] is a female predominant liver-specific autoimmune disease with middle-age onset. It has an average incidence of 2.7 cases per 100000[34], but epidemiological studies suggest that the incidence of PBC is increasing[35]. There is variation in the prevalence of disease between geographic locations[35,36]; PBC is more prevalent in Northern Europe, North America and Latin America and less common in Eastern Asia, Africa, and Australia[37,38]. Clinically, PBC is characterized by the presence of high titer AMA and immune-mediated progressive destruction of biliary epithelial cells (BECs) within small bile ducts, eventually leading to cholestasis, fibrosis, and, potentially, liver cirrhosis[12]. Approximately 50%-60% of patients are asymptomatic at diagnosis. The disease has a long latency period[39,40], followed by the development of symptoms that may include fatigue, pruritus, cutaneous pigmentation and, later, bleeding varices, edema, or ascites[41]. The prognosis of patients diagnosed with PBC has improved significantly over the past two decades, perhaps because patients are being diagnosed earlier. PBC is a "model" autoimmune disease with significant literature on genetics, environment and animal models[17,25,33,42-51].

The female predominance among individuals with PBC suggests that there are significant genetic components in this disease, supported by the high frequency of X chromosome monosomy in patients with PBC[52,53] and Y chromosome loss in male patients with PBC[54]. Reports from recent genetic studies demonstrate that in addition to the MHC, several loci are associated with susceptibility to PBC, including interleukin (IL) 12-related pathways, SPIB, IRF5-TNPO3, and 17q12-2. The candidate genes identified by genome wide association studies include STAT4, DENND1B, CD80, IL7R, CXCR5, TNFRSF1A, CLEC16A, and NFKB1[55-58]. Data on familial clustering of PBC demonstrates that first-degree relatives of PBC patients have an increased risk of developing disease and most often these clusters involve mother-daughter pairs, consistent with the female preponderance of the disease[59-61]. Furthermore, twin studies have demonstrated a high concordance for PBC in monozygotic twins[62]. These studies provide evidence for a genetic basis underlying PBC. Genome analysis of DNA methylation, copy number variation and gene expression of monozygotic twins and sisters discordant for PBC have also indicated a contribution of epigenetic events[63]. However, environmental factors also play a role in the development of the disease[64], and multiple environmental components including chemicals[30,65-67] and bacteria[68-71] have been implicated.

IMMUNOLOGICAL FEATURES OF PRIMARY BILIARY CHOLANGITIS

AMA are present in over 95% of patients with PBC and are diagnostic of PBC[23]. The autoantigens of AMA have been identified as the E2 subunits of the 2-oxo-acid dehydrogenase complexes (2OADC-E2), including the E2 subunits of the pyruvate dehydrogenase complex (PDC-E2), branched chain 2-oxo acid dehydrogenase complex (BCOADC-E2) and 2-oxo-glutarate dehydrogenase complex (OGDC-E2) within the inner mitochondrial matrix[13,15,16,72]. The E2 enzymes have a common structure consisting of an N-terminal domain containing a single or multiple lipoyl groups. Previous studies have demonstrated that the dominant epitopes

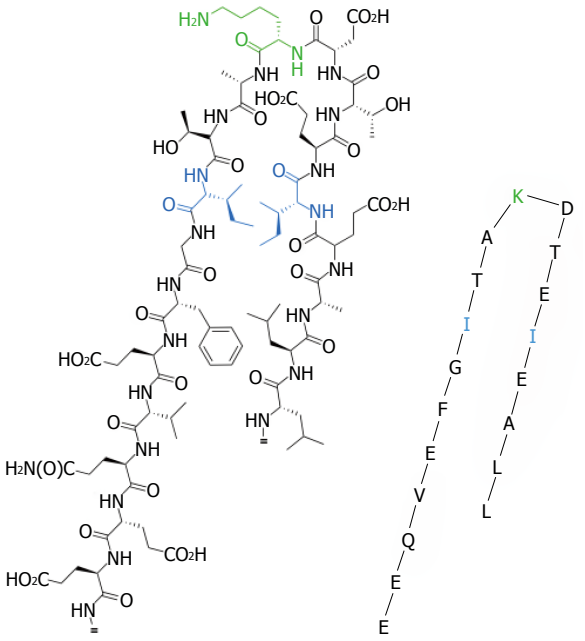

Figure 1 Schematic representation of the pyruvate dehydrogenase E2 lipoyl domain. Including the 19 residues (LLAEI-ETDKA-TIGFE-VQEE), lipoic acid is covalently attached to the ε group of lysine (K) *via* an amide bond.

recognized by AMA are all within the lipoyl domains of these target antigens[73].

In patients with PBC, Both $CD4^+$ and $CD8^+$ T cells are present in portal tracts, around damaged bile ducts, strongly suggesting the participation of cellular immune mechanisms in biliary damage[12]. PDC-E2 autoreactive CD4 T cells are present in peripheral blood and liver; there is a specific 100-150 fold increase in the number of PDC-E2-specific CD4 T cells in the hilar lymph nodes and liver versus peripheral blood in patients with PBC[27]. The PDC-E2 autoepitope for both CD4 and HLA class Ⅰ restricted CD8 T cells, overlaps with the B cell epitope, which spans the lipoyl domain[74]. Similar to CD4 autoreactive T cells, there is a 10-fold higher frequency of PDC-E2 specific CD8 T cells within the liver versus peripheral blood. Moreover, the precursor frequency of PDC-E2-specific autoreactive CD8 T cells is significantly higher in early rather than late stage of the disease[74]. Recent reports also substantiate the significance of innate immunity, including monocytes, toll like receptors and natural killer cells in the development of PBC[75,76]. The multilineage response to the PDC-E2, the immunodominant mitochondrial autoantigen in PBC, points to the thesis that loss of tolerance to PDC-E2 is the initiating event that leads to the subsequent development of clinical biliary pathology[12].

MOLECULAR MIMICRY OF LIPOIC ACID AND XENOBIOTICS IN PBC

Epidemiological and mechanistic studies on autoimmunity have strongly demonstrated the etiologic contribution of environment[77], likely through molecular mimicry. Although microorganisms are possible candidates for the induction of autoimmune disease by molecular mimicry[78-84], there are other potential environmental factors, including chemical compounds foreign to a living organism. Examples include drugs, pesticides or other organic molecules that have the potential to modify host proteins and render them more immunogenic[77].

Halothane hepatitis is a xenobiotic-induced liver disease that occurs when susceptible individuals develop an immune response against trifluoroacetylated (TFA)-adduct protein. Exposure to TFA-conjugated self proteins results in antibody responses against such TFA-self proteins. Interestingly, anti-TFA also recognizes the lipoylated domain of PDC-E2[14,85]. The immunological cross-reactivity of anti-TFA antibodies with the immunodominant epitope in PBC prompted us to examine in depth molecular mimicry.

Site-directed mutagenesis of the PDC-E2 lipoyl domain demonstrated that AMA recognition is constrained by respective amino acid sequence in epitope (Figure 1, Table 1)[86,87]. The uniqueness of epitope specificity of AMA within the lipoyl domains of the 2OADC-E2 enzymes in patients with PBC[87-89] suggests that the lipoic acid domain is likely a lynchpin to the etiology of PBC. High resolution structural analysis and modeling studies of the PDC-E2 lipoyl domains from both prokaryotes and eukaryotes demonstrates that lipoic acid is covalently attached to the ε group of lysine (K) *via* an amide bond and is prominently displayed on the outer surface of PDC-E2. More importantly, the ability of lipoic acid to rotate by means of its "swinging arms" with respect to the bulk of the entire PDC-E2 molecule allows accessibility of its dithiolane ring for reduction acylation[90,91]. Although the change in conformation and the existence of multiple conformations of the lipoyl domain during reductive acetylation are important in catalyzing acyl transfer[90], it also renders PDC-E2 susceptible to aberrant chemical modifications.

Accumulating evidence implicates that the loss of tolerance to PDC-E2 is pivotal in the initiation event of PBC and that AMA specificities reflect aspects of the induction phase of the disease[11,25,31,39]. Indeed the role of environment is well-known in many autoimmune diseases[30,92-98].

We hypothesized that xenobiotic modification of the native lipoyl moiety of the major mitochondrial autoantigen PDC-E2, may lead to loss of self-tolerance and eventually biliary lesions (Figure 2)[99]. This thesis is based on the findings of (1) readily detectable levels of immunoreactivity of PBC sera against comprehensive panels of protein microarrays, which mimic the inner lipoyl domain of PDC-E2; and (2) subsequent quantitative structure-activity relationships. Data from quantitative structure-activity relationship (QSAR) analysis demonstrated that AMA-

Table 1 Serological reactivity of primary biliary cholangitis sera to the recombinant proteins of wild type pyruvate dehydrogenase E2 lipoyl domain, single amino acid mutants double, triple and quadruple mutants[1]

Mutant No.		Amino acid sequence	PBC sera[2]		Purified PBC IgG[3]
			IgG	IgM	
PDC-E2 wild type		LLAEIETDKATIGFEVQEE	1	1	1
Mutant 3		LLAEAETDKATIGFEVQEE	0.476 ± 0.029	0.504 ± 0.043	0.408 ± 0.052
Mutant 9		LLAEIETDKATAGFEVQEE	0.706 ± 0.029	0.781 ± 0.054	0.552 ± 0.065
Mutant 12		LLAEIETDKATIGFAVQEE	0.659 ± 0.034	0.768 ± 0.096	0.482 ± 0.074
Double amino acid substitution	Mutant 1	ALAEIETDKATAGFEVQEE	0.334 ± 0.029	0.253 ± 0.034	0.075 ± 0.023
	Mutant 2	ALAEIETDKATIGFAVQEE	0.461 ± 0.031	0.435 ± 0.045	0.663 ± 0.069
	Mutant 3	LLAEAETDKATAGFEVQEE	0.066 ± 0.009	0.093 ± 0.016	0.024 ± 0.007
	Mutant 4	LLAEAETDKATIGFAVQEE	0.111 ± 0.017	0.095 ± 0.016	0.043 ± 0.016
Triple amino acid substitution	Mutant 1	ALAEAETDKATAGFEVQEE	0.017 ± 0.004	0.044 ± 0.009	0.038 ± 0.017
	Mutant 2	LLAEAETDKATAGFAVQEE	0.019 ± 0.003	0.054 ± 0.012	0.050 ± 0.007
Quadruple amino acid substitution		ALAEAETDKATAGFAVQEE	0.024 ± 0.005	0.066 ± 0.012	0.075 ± 0.031

[1]16 single alanine substitution mutants along a peptide that constitutes the beta sheet of the PDC-E2 inner lipoyl domain, 4 double aa substitution mutants, 3 triple and one quadruple mutants were also constructed. Purified proteins from all these constructs were analyzed for Ig reactivity with PBC sera. 3/16 of the single amino acid mutants have much reduced antibody binding are shown. Other alanine substitutions have Ig reactivity similar to wild type PDC-E2. Control sera samples include (lupus, n = 30, Crohn's disease, n = 20, PSC, n = 28, scleroderma n = 20) did not react; [2]Relative ratio of serological IgG and IgM reactivity compared to wild type determined by ELISA at 1:4000 sera dilution (n = 60); [3]Relative ratio of purified IgG reactivity to wild type determined by ELISA (n = 10). PBC: Primary biliary cholangitis.

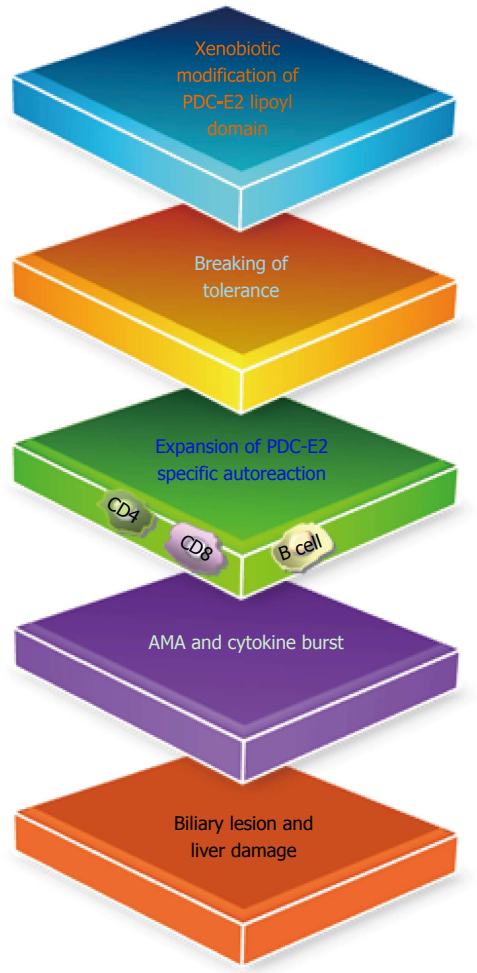

Figure 2 Xenobiotic modification of the native lipoyl moiety of the major mitochondrial autoantigen pyruvate dehydrogenase E2, lead to the loss of self-tolerance and eventually biliary lesions in primary biliary cholangitis. PDC-E2: Pyruvate dehydrogenase E2; AMA: Anti-mitochondrial autoantibody.

positive PBC sera, but not controls, reacted to a number of xenobiotic-modified PDC-E2 structures[66,100] with a particularly striking level of reactivity against 6,8-bis(acetylthio) octanoic acid (SAc)-PDC-E2[101]. Recent data further suggest that chemical modification of PDC-E2 lipoic acid, *via* an electrophilic attack on the lipoic acid disulfide bond, triggers loss of tolerance to PDC-E2[30,101,102]. Such modifications could substantially affect the conformation of the PDC-E2 lipoyl domain and its immunogenicity in genetically susceptible hosts. Importantly, one of these chemical compounds is 2-octynoic acid (2-OA), a chemical commonly found in cosmetics and food additives[66].

XENOBIOTICS INDUCED MODELS OF PBC AND THE CONTRIBUTIONS OF EFFECTOR PATHWAYS IN AUTOIMMUNE CHOLANGITIS

Interestingly, immunization of C57BL/6 mice and NOD.1101 (NOD.B6 *Idd10 Idd18*r2) mice with 2-OA coupled to BSA, but not BSA alone, induced high titer AMAs, portal inflammation, and autoimmune cholangitis similar to human PBC[103,104]. These models provide a persuasive argument in favor of an environmental origin for human PBC[81,103,105,106]. We further investigated the role of IL-12-Th1/IL-23-Th17 pathways in the development of autoimmune cholangitis in this PBC model by using specific cytokine knockout mice (Table 2)[18]. In particular, we constructed several unique gene-deleted mice, including C57BL/6 mice deleted in both Th1 and Th17 (IL-12p40), Th1 cytokine (IL-12p35, IFN-γ) or Th17 cytokine (IL-23p19, IL-17A, IL-

Table 2 Influence of Th1 and Th17 cytokine in liver pathology of 2OA-BSA induced primary biliary cholangitis mouse model

Pathway	Cytokine k/o	Liver pathology
Th1	IL-12p35$^{-/-}$	Reduced liver infiltrates, reduced bile duct damage
Th1	IFN-γ$^{-/-}$	Marked reduction in liver infiltrates, bile duct normal
Th1/Th17	IL-12/IL-23p40$^{-/-}$	Abolish autoimmune cholangitis
Th17	IL-23p19$^{-/-}$	Reduced liver infiltrates, reduced bile duct damage
Th17	IL-17A$^{-/-}$	Reduced liver infiltrates, reduced bile duct damage
Th17	IL-17F$^{-/-}$	Similar to positive control
Th17	IL-22$^{-/-}$	Reduced liver infiltrates, reduced bile duct damage

IFN-γ: Interferon-γ; IL: Interleukin; Th17: T helper 17.

17F or IL-22). We immunized each of these cytokine-deficient mice with 2-OA-BSA and followed the natural history of their immunopathology. Our data indicate that while both IL-12/Th1 and IL-23/Th17 are involved in cholangitis, it is the IL-12/Th1 signaling pathway that elicits liver pathology in this xenobiotic induction disease model of PBC. In fact, deletion of IFN-γ prevents disease and suppresses autoantibodies. Importantly, deletion of the Th17 cytokines IL-17A and IL-22, but not IL-17F, reduces biliary damage; IL-17A-knockout mice have also reduced levels of AMAs. We further demonstrated that the production of IFN-γ is significantly decreased in livers of IL-23p19$^{-/-}$, IL-17A$^{-/-}$ and IL-22$^{-/-}$ mice compared with controls. However, the ability of T cells to produce IFN-γ was not affected in Th17 cytokine-deficient mice. Thus, in the 2-OA-BSA immunized mice model: (1) Both IL-12/Th1 and IL-23/Th17 are involved in cholangitis; (2) IL-12/Th1 signaling pathway is critical in eliciting liver pathology; and (3) IL-23/Th17 pathway is involved in perpetuating the IL-12/IFN-γ mediated pathology. We also investigated the role of B cells in the pathogenesis of PBC by depleting B cells using two different monoclonal antibodies, CD20 and CD79. B cell depletion led to exacerbated cholangitis, with higher T cell infiltrates and inflammatory cytokines, indicating a protective role of B cells in PBC[107].

2OA-BSA immunized C57BL/6 mice were also studied for the potential of CTLA4-based therapy on cholangitis by using CTLA4-Ig. CTLA4-Ig is a soluble recombinant human fusion protein comprised of the extracellular domain of human CTLA4 linked to a modified portion of the Fc domain of human IgG[108,109]. In mice treated begun one day before 2-OA-BSA immunization, CTLA4-Ig completely inhibits the manifestations of cholangitis, including AMA production, intra-hepatic T cell infiltrates and bile duct damage. However, treatment with CTLA-4 Ig initiated after the development of autoimmune cholangitis in 2OA-BSA immunized mice, reduced intra-hepatic T cell infiltrates and biliary cell damage, although AMA levels were not altered[110].

We also investigated the role of innate immunity and natural killer T (NKT) cells on modulating disease activity in this xenobiotic-induced mouse model. Briefly, we immunized mice with and without the addition of α-galactosylceramide (α-GalCer), an invariant natural killer T cell activator. 2-OA-BSA-immunized mice exposed to α-GalCer developed a profound exacerbation of their autoimmune cholangitis, including significant increases in CD8$^+$ T cell infiltrates, portal inflammation, granuloma formation, and bile duct damage. Moreover, these mice produced increased levels of AMAs and evidence of fibrosis[111]. CD4 and CD8 knock-out mice immunized with either 2-OA-BSA/PBS or 2-OA-BSA/α-GalCer develop AMAs and portal infiltrates. However, 2-OA-BSA/α-GalCer treated mice also develop fibrosis. Indeed, our data suggest that innate immunity is critical for immunopathology and that the pathology is exacerbated in the presence of α-GalCer[50]. More recently, we also reported that 2-OA-BSA-immunized mice administered with a Th2-biasing agonist (2s,3s,4r)-1-*O*-(a-D-galactopyranosyl)-*N*-tetracosanoyl-2-amino-1,3,4-nonanetriol (OCH), developed portal inflammation and hepatic fibrosis similar to mice treated with α-GalCer[75]. However, inflammatory portal cell infiltrates and AMA responses are reduced in iNKT cell deficient CD1d knockout mice treated with OCH. These results suggest that activation iNKT cells can occur *via* overlapping and/or promiscuous pathways and further highlight the role of innate immunity in the natural history of PBC.

Our data also provides clues to the mechanisms by which autoimmune diseases could be perpetuated in humans and also helps explain recurrence of PBC following liver transplantation in the absence of major histocompatibility complex (MHC) compatibility matching. Thus, in the absence of MHC restriction, disease reoccurrence would depend on a non MHC restricted cellular mechanisms, suggesting that biliary epithelial cells are simply an innocent victim of an immune attack. Thus, they attract immune attack by virtue of their unique biochemical mechanisms by which they process PDC-E2 during apoptosis[20]. Bile duct cells may have a direct effector role in immune-mediated cholangiopathies and fibrosis through their own cellular senescence pathway[112]. This also explains the suggested success of ursodiol in PBC, a drug that appears to have anti-apoptotic properties and also may modulate innate responses. Our data would also explain the relative failure of immunosuppressive drugs to alter PBC, because such agents are relatively ineffective against innate mechanisms. Finally, the induction of fibrosis in 2-OA-BSA-immunized mice exposed to α-GalCer permits not only dissection of its induction, but also has the potential to be useful in studies of intervention.

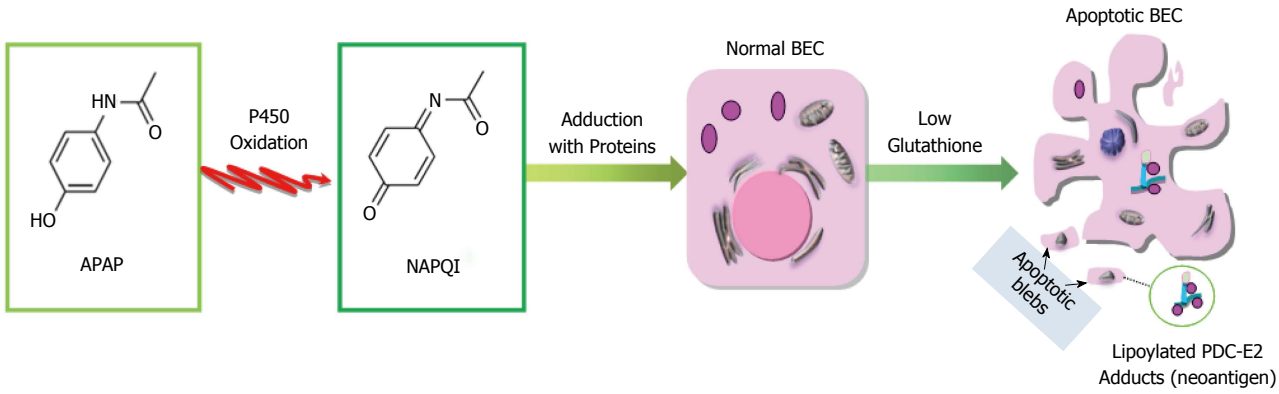

Figure 3 APAP metabolism and proposed mechanism of APAP-mediated breaking of immune tolerance. APAP is metabolized in the liver to nontoxic compounds *via* conjugation of the aromatic ring to sulfate or glucuronic acid. APAP is converted into a highly electrophilic metabolite, NAPQI by microsomal cytochrome P450 oxidation. Reactive NAPQI accumulates and can form adducts with cellular proteins, leading to disruption of cellular functions, generation of neo-antigens, and loss of tolerance.

LINK BETWEEN XENOBIOTICS AND AMA IN ACETAMINOPHEN INDUCED LIVER INJURIES

Although it is not clear how xenobiotics or the modified cellular proteins initiate autoimmunity in PBC, analysis of serum samples from subjects with acute liver failure indicate that a severe liver oxidant injury could lead to AMA production[113]. Specifically, 217 serum samples from 69 patients with acute liver failure (ALF) collected up to 24 mo post-ALF were compared with controls, for titer and reactivity with 2OADC-E2. AMA were detected in 28/69 (40.6%) ALF patients with reactivity found against all of the major mitochondrial autoantigens. The strikingly high frequency of AMAs in patients with ALF supports the thesis that oxidative stress-induced liver damage may lead to AMA induction. In particular, we note that AMA with the same antigen and epitope specificity as in patients with PBC was found in almost 35% of the acetaminophen (or APAP, chemically named N-acetyl-p-aminophenol) poisoning subjects, suggesting that the PDC-E2 lipoyl domain is likely a target of APAP induced reactive oxygen species. This finding is of significance as toxic doses of APAP produces reactive oxygen and nitrogen species and reactive metabolites[114-117] that could result in mitochondrial damage and liver injury as evidenced by the elevation of serum alanine amino transferase and P450 dependent centrilobular damage[118,119].

APAP is the most widely used non-prescription drug in the United States. Using the recommended therapeutic dosage (1000 mg per single dose and up to 4000 mg per day for adults), 85% of acetaminophen is metabolized in liver to non-toxic compounds *via* the conjugation of the aromatic ring to sulfate or glucoronic acid. The remaining 15% is converted into a highly-electrophilic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) through isozymes of microsomal cytochrome P450[120]. In the presence of the reduced form of glutathione (GSH), NAPQI can either be covalently linked to GSH *via* Michael's addition to the aromatic ring or reduced back to APAP[121]. The predominant method of NAPQI detoxification occurs through the former mechanism, resulting in depletion of the intracellular glutathione pool[122]. However, in the presence of excess APAP or when microsomal P450 is increased, hepatic GSH is depleted more extensively and cannot compete efficiently with the increased NAPQI. The resulting decrease in cellular glutathione further allows the accumulation of reactive NAPQI, which then reacts with nucleophilic sites such as cysteine and lysine residues on cellular proteins and related cofactors[123].

Previous data[124] have suggested that glutathiolation decreases the antigenicity of PDC-E2. Due to cellular depletion of glutathione, very little glutathione would be available for such covalent protection of PDC-E2. The depletion of glutathione could lead to neo-antigens through modification of native PDC-E2 by high levels of reactive NAPQI or other electrophilic agents. We reason that in PBC such electrophilic modification on lipoic-acid-conjugated PDC-E2 will inhibit the physiological function of PDC-E2 and subsequently lead to disruption of ATP synthesis, cell death and the release of either unmasked PDC-E2 or neoantigens formed by xenobiotics-modified PDC-E2. Microarray studies on APAP toxicity also revealed consistent altered transcriptome expression in oxidative phosphorylation, protein post-translational modification in liver and blood samples[125,126]. The exposure of this chemical modified self-protein to the immune system of genetically susceptible individuals could lead to the breakdown of self-tolerance to native PDC-E2 itself by molecular mimicry and epitope spreading mechanism. Thus, in genetically susceptible individuals, the prolonged exposure to electrophilic agents, such as acetaminophen may initiate and/or enhance the breakdown of self-tolerance to PDC-E2 and eventually lead to PBC (Figure 3).

CONCLUSION

The etiological mechanism of the immunological specificity of the 2-OADC-E2 enzymes lipoyl domain in PBC remains an enigma. Recent quantitative structure-activity relationship (QSAR) studies suggest that disruption of the lipoyl ring S-S linkage renders the lipoic acid "activated" and receptive for xenobiotic modification and subsequent AMA recognition[101]. Data from immunological characterization of antigen and Ig isotype specificities against one such lipoyl acid mimic SAc and rPDC-E2 strongly support a xenobiotic etiology in PBC. This observation is of significance in light of the high frequency of AMAs in patients with ALF. In particular, AMA was found in almost 35% of APAP poisoning subjects in a cohort of ALF patients[113]. Highly reactive electrophilic metabolites of APAP such as NAPQI can deplete the intracellular glutathione pool and render PDC-E2 vulnerable to further modification by electrophiles. Such mechanisms of *in vivo* generation of xenobiotic modified self proteins could lead to the breaking of tolerance to native proteins through molecular mimicry and antigen spreading in genetically susceptible individuals[102]. Finally, the recapitulation of AMA and PBC-like biliary lesions in 2OA-BSA immunized mice further support our working hypothesis on xenobiotic etiology of PBC[103]. Future work is directed at examining the biochemical and immunological mechanisms underlying the breach of tolerance in autoimmunity in PBC by environmental chemicals. Knowledge gained from this model may have significant preventive and therapeutic implications in the clinical management of PBC.

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REVIEW

Combination antiretroviral studies for patients with primary biliary cirrhosis

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Abstract

Following the characterization of a human betaretrovirus in patients with primary biliary cirrhosis (PBC), pilot studies using antiretroviral therapy have been conducted as proof of principal to establish a link of virus with disease and with the eventual aim to find better adjunct therapies for patients unresponsive to ursodeoxycholic acid. In the first open label pilot study, the reverse transcriptase inhibitor lamivudine had little demonstrable biochemical or histological effect after 1 year. Whereas, lamivudine in combination with zidovudine was associated with a significant reduction in alkaline phosphatase as well as improvement in necroinflammatory score, cholangitis and ductopenia over a 12 mo period. A double blind, multi-center randomized controlled trial using lamivudine with zidovudine for 6 mo confirmed a significant reduction in alkaline phosphatase, ALT and AST in patients on antiviral therapy. However, none of the patients achieved the stringent endpoint criteria for normalization of alkaline phosphatase. Furthermore, some patients developed biochemical rebound consistent with drug resistance. A major fault of these studies has been the inability to measure the viral load in peripheral blood and therefore, provide a direct correlation between improvement of hepatic biochemistry and reduction in viral load. Nevertheless, viral mutants to lamivudine with zidovudine were later characterized in the NOD.c3c4 mouse model of PBC that has been used to test other antiretroviral regimens to betaretrovirus. The combination of tenofovir and emtricitabine reverse transcriptase inhibitors and the HIV protease inhibitor, lopinavir were found to abrogate cholangitis in the NOD.c3c4 mouse model and the same regimen normalized the liver tests in a PBC patient with HIV and human betaretrovirus infection. This combination antiretroviral therapy has now been used in a double blind randomized controlled crossover study for patients with PBC followed by an open label extension study. Only a third of the PBC patients were able to tolerate

the lopinavir but those maintained on tenofovir, emtricitabine and lopinavir experienced sustained and clinically meaningful reduction in hepatic biochemistry. While we await the histological and virological evaluation, it is clear that better tolerated regimens of antiretroviral treatment will be required in future clinical trials.

Key words: Primary biliary cirrhosis; Antiretroviral therapy; Human betaretrovirus; Randomized controlled trial; Endpoints for trials

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Core tip: Early experience with antiretroviral therapy in primary biliary cirrhosis (PBC) patients strongly suggests that reverse transcriptase inhibitors alone lack efficacy to provide sustained and clinically meaningful biochemical responses. In contrast, combination antiretroviral therapy with human immunodeficiency virus protease inhibitors have been linked with robust and long-lived biochemical responses in PBC patients capable of tolerating the therapy. The use of digital droplet polymerase chain reaction has markedly improved the sensitivity of viral detection in peripheral blood and should enable studies to link reduction in viral load with improvements in hepatic biochemistry and histology.

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INTRODUCTION

In 2003, our group characterized a human betaretrovirus (HBRV) in patients with primary biliary cirrhosis (PBC) as part of an international collaboration. The discovery of a virus in PBC was unexpected because most researchers at the time were studying the role of molecular mimicry of bacterial proteins with mitochondrial antigens[1]. Although studies have linked infection with a disease specific mitochondrial phenotype in cell culture and in mouse models of PBC, we currently lack definitive ways to prove a causal association between HBRV infection and PBC[2]. Indeed, it is challenging to provide proof of any infectious process in a complex and chronic disease with genetic predisposition[3]. Accordingly, there is a justification for performing interventional studies aimed at investigating a causal association between microbe and disease[4,5]. One of our clinical objectives, therefore, has been to conduct a proof of principal interventional study to link HBRV infection with PBC[6] and to identify safe and efficacious antiviral regimens for patients with PBC[7,8].

At the time we initiated pilot clinical studies prior to 2003, lamivudine was commonly used to treat patients with hepatitis B virus infection[9]. As this reverse transcriptase inhibitor had broad coverage and a proven safety record in patients with liver disease, we embarked on the first antiviral study in patients with PBC. We subsequently progressed to using dual reverse transcriptase inhibitors[9,10] and then HIV protease inhibitors[11], once we had demonstrated utility of these regimens *in vitro* and in mouse models of PBC[8]. Herein, we give an overview of the investigation of antiretroviral activity against betaretroviruses and our experience to date in treating patients with PBC with antiviral therapy.

DISCOVERY OF THE HUMAN BETARETROVIRUS IN PRIMARY BILIARY CIRRHOSIS

In the years running up to 2003, several exploratory studies were conducted looking for potential environmental triggers for PBC. We found no evidence of bacterial infection in PBC patients livers using 16s RNA PCR and turned to the subtractive hybridization methodology, representational difference analysis to uncover viral sequences in a PBC patient's liver[12]. Follow up studies were performed to demonstrate serum reactivity to viral proteins in PBC patients serum using Western blots[13] and virus-like particles in biliary epithelium isolated from PBC patients by electron microscopy[14]. Then an unbiased approach was employed using consensus PCR primers capable of amplifying retroviral *pol* gene sequences to identify a betaretrovirus *pol* sequence. The full-length virus was cloned from PBC patient samples that shared marked nucleotide similarity with the mouse mammary tumor virus (MMTV), a betaretrovirus associated with breast cancer in mice[14,15]. The HBRV was also found to have 97% identity with human mammary tumor virus sequences found in human breast cancer samples[16,17]. The agent was referred to as HBRV because of the similarity with the mouse betaretrovirus, MMTV[14-16]. HBRV is an exogenous virus that is not encoded within the human genome as an endogenous retrovirus. Whereas MMTV is encoded in the genome of most mice and infection can be acquired from an exogenous source, such as breast milk or from an endogenously expressed provirus[18]. At present, it is not known whether HBRV infection in humans is passaged as a zoonosis from mice or acquired as a result of spread from other infected individuals.

ROLE OF HUMAN BETARETROVIRUS IN PRIMARY BILIARY CIRRHOSIS

The role that HBRV plays in the pathogenesis of PBC is still debated[2,19]. In early studies, the virus was

predominantly detected in lymph nodes rather than in the liver, similar to observations of MMTV infection in mice[20]. Approximately 75% of peri-hepatic lymph node samples derived from PBC patients at the time of liver transplantation were positive for HBRV protein and RNA, whereas only 1 in 3 PBC patients had detectable HBRV RNA in the liver[14]. Other groups experienced difficulty with detection virus in the liver. For example, one lab was unable to detect viral DNA in PBC liver using a single round of PCR and a separate group found HBRV in 5% of patients with PBC during a survey of liver disease patients for infection[21]. In agreement, our lab rarely found hepatic HBRV DNA (about 5%) using nested-PCR. Taken together, these studies are concordant and suggest that more sensitive techniques have a higher detection rate in different tissue compartments[4,14]. Nevertheless, the perceived lack of detection of HBRV at the site of disease has caused considerable controversy and confusion[21,22]. Indeed, Selmi *et al*[22] suggested, "In our opinion, the only possible final evidence for a role of a beta-retrovirus in PBC could be provided by the direct demonstration, possibly through chromatograms, of the insertion of viral sequences in the genome of a large number of patients with PBC".

It is generally agreed that the detection of proviral integrations is considered the gold standard to confirm retroviral infection. To address this issue, ligation mediated-polymerase chain reaction (PCR) was used to identify the junction regions where the betaretroviral long terminal repeat joins up with the human genome. Next generation sequencing was employed to characterize the proviral integrations and increase the sensitivity of the reactions. In these studies, HBRV proviral integrations and HBRV RNA were detected in two thirds of PBC patients' biliary epithelium samples[23]. Viral integrations studies also established the presence of HBRV in PBC patients' lymph nodes, whereas integrations were rarely observed in the liver, in keeping with clinical observations from most laboratories. *In vitro* studies confirmed that PBC patients' lymph nodes harbored infectious virus following the isolation of the HBRV in cell culture[24]. Taken together, these data suggest that HBRV can be found at the site of disease and isolated from patients with PBC.

The prevalence studies also revealed the presence of HBRV in patients without PBC, bringing up the concern with lack of specificity. In our viral integration studies, infection was commonly found in patients with cryptogenic liver disease and autoimmune hepatitis (AIH) as well as a small a proportion of control samples[23]. We had previously observed HBRV in patients with AIH[25], which is consistent with the knowledge that up to 20% of patients with PBC have overlap features with AIH[26,27]. These data suggest a hypothesis that HBRV may be associated with different phenotypic manifestations of liver disease modulated by genetic and other factors. However, another lab using nested PCR found HBRV in patients with various hepatic diagnoses - but not healthy controls[21]. If HBRV infection is associated with the development of liver disease *per se*, these data could be compared with early observations following the discovery of hepatitis C virus. Viral infection was not just confined to those with blood transfusions and high risk behavior but also found in patients with various diagnostic categories, such as alcoholic liver disease, hepatitis B virus co-infection, autoimmune hepatitis and cryptogenic cirrhosis to name a few. Another consideration is that better diagnostic methods will be required to determine the true prevalence of HBRV infection in patients and healthy subjects as PCR studies can be prone to artifact.

It is important to emphasize that the association of HBRV with PBC does not imply causation as many further layers of proof are required to support an etiological role for virus and in the disease process[2]. In this regard, it is interesting that HBRV has been linked with a disease specific phenotype of PBC. It is thought that patients with PBC make anti-mitochondrial antibodies (AMA) because the mitochondrial antigen that reacts with AMA is found exposed on the cell surface of biliary epithelium and peri-hepatic lymph nodes[28]. This in turn leads to the loss of tolerance to self-proteins. HBRV is implicated in the process because viral proteins have been found in the same cells that have the aberrant mitochondrial protein expression in PBC patients' lymph nodes[14]. As lymph nodes are a major reservoir for HBRV in humans, we used lymph nodes homogenates to construct an *in vitro* transmission model of PBC. In these studies, the homogenates were co-cultured with normal biliary epithelial cells that developed cell surface expression of the AMA reactive mitochondrial proteins[29]. Subsequently, pure isolates of MMTV and HBRV were shown to promote the PBC phenotype, whereas control viruses did not. Taken together, these studies provide a theoretical model for the viral induction of autoimmunity where the virus triggers an immune response to viral proteins but at the same time the lymphocytes respond to self antigens that are usually hidden within the cells[1].

ROLE OF MOUSE MAMMARY TUMOR VIRUS IN AUTOIMMUNE BILIARY DISEASE

MMTV has also been linked with a disease specific phenotype of PBC in mice models of autoimmune biliary disease[8,30]. These PBC mouse models are mainly derived from immunodeficient mice that develop spontaneous liver disease with AMA production[3,31]; they include the NOD.c3c4 mouse[32,33], IL-2 receptor $\alpha^{-/-}$[34], T cell TGF-β receptor Ⅱ dominant-negative (dnTGFβRⅡ)[35], and Scurfy mouse lacking T regulatory cells[36]. The NOD.c3c4 mouse has several

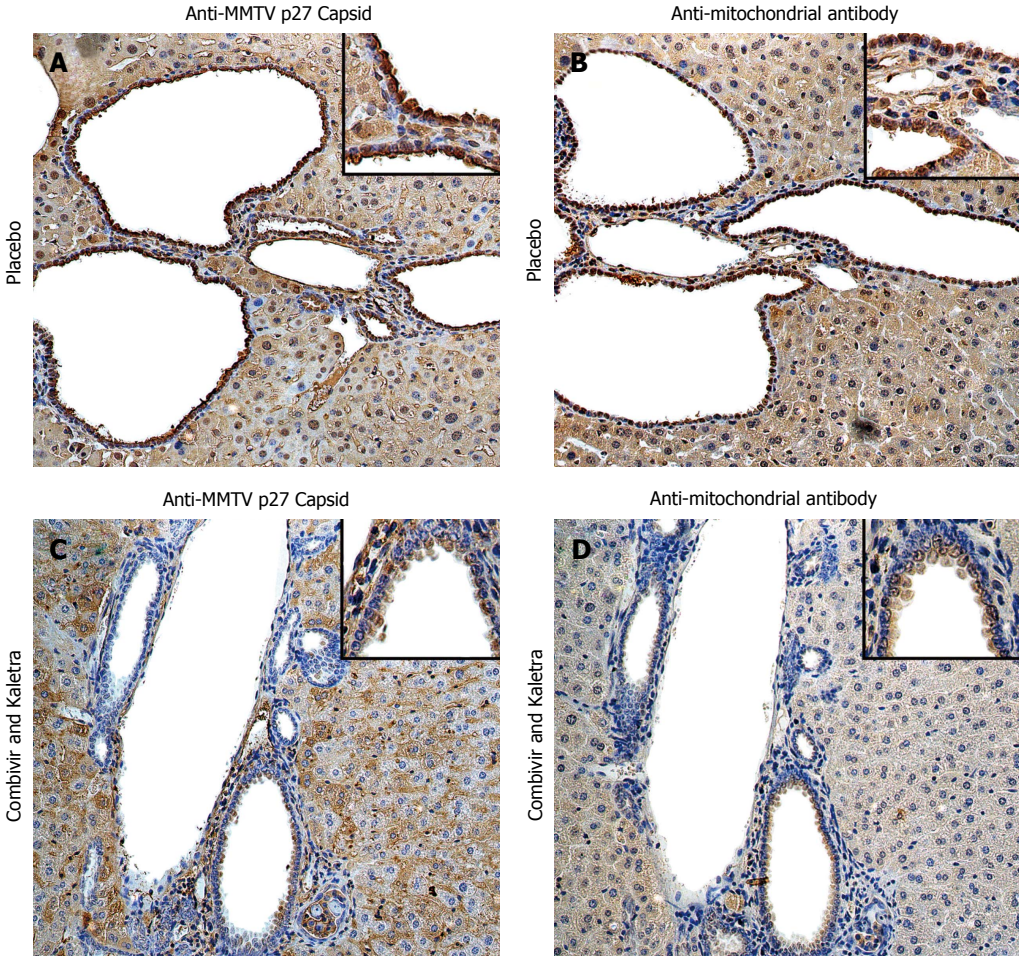

Figure 1 Hepatic immunohistochemistry study of NOD.c3c4 mice treated with placebo (A and B) or tenofovir/emtricitabine and lopinavir/ritonavir (C and D) for 12 wk. A: Mice receiving placebo showed anti-MMTV p27 Capsid reactivity in biliary epithelial cells and hepatocyte nuclei to a lesser extent; B: The distribution of anti-mitochondrial antibody staining was observed in a similar biliary distribution pattern on the bile duct epithelium; C and D: The reactivity to both viral and mitochondrial proteins was attenuated in mice treated with tenofovir/emtricitabine and lopinavir/ritonavir. Haematoxylin eosin staining, magnification × 2009 with × 4009 in insets showing staining in biliary epithelial cells. With permission from Sharon *et al*[8].

features of PBC with progressive granulomatous cholangitis and liver failure, whereas the other models die from multi-organ disease[36-39]. However, the NOD.c3c4 mouse also has features inconsistent with PBC, such as cystic dilatation of bile ducts (Figure 1).

MMTV is common in laboratory mice and the frequent appearance of AMA in immunodeficient mice suggested a hypothesis that disease was being triggered by MMTV[31]. The viral infection may be acquired from an endogenous MMTV provirus source or from an exogenous infection passaged in milk[18]. Indeed, it is known that endogenous retroviruses can recombine and emerge as pathogens in mice with defective innate and adaptive immune responses[40,41]. Evidence for a similar process was found in autoimmune biliary disease mouse models, where MMTV infection was located in lymphoid tissues that also expressed AMA reactive protein[30]. The NOD.c3c4 had evidence of MMTV proteins in the bile ducts associated with mitochondrial protein expression (Figure 1A and B). Moreover, the NOD.c3c4 mice were found to develop contemporaneous humoral immune responses to MMTV with AMA production[30]. These data are consistent with the model that betaretrovirus infection triggers autoantigen expression that in turn, breaks tolerance to self-antigens[1].

Using mouse models to test combination antiretroviral therapy against betaretrovirus

The NOD.c3c4 model has been treated with antiretroviral therapy to investigate whether MMTV is implicated in the development of autoimmune biliary disease. It has been established that MMTV is sensitive to the HIV reverse transcriptase inhibitors zidovudine[42] and tenofovir[43] and the HIV protease inhibitor lopinavir[7] *in vitro*. Accordingly, the *in vivo* mouse model studies have served the dual purpose for examining whether specific antiviral regimens may be useful for testing in translational studies for patients with PBC as well.

For the NOD.c3c4 studies, mice were treated from age 8 weeks to 20 wk and evaluated for reduction in alkaline phosphatase (ALP), hepatic MMTV levels as well as liver histology using the Ishak score. Up to 20 mice per group were treated with the reverse

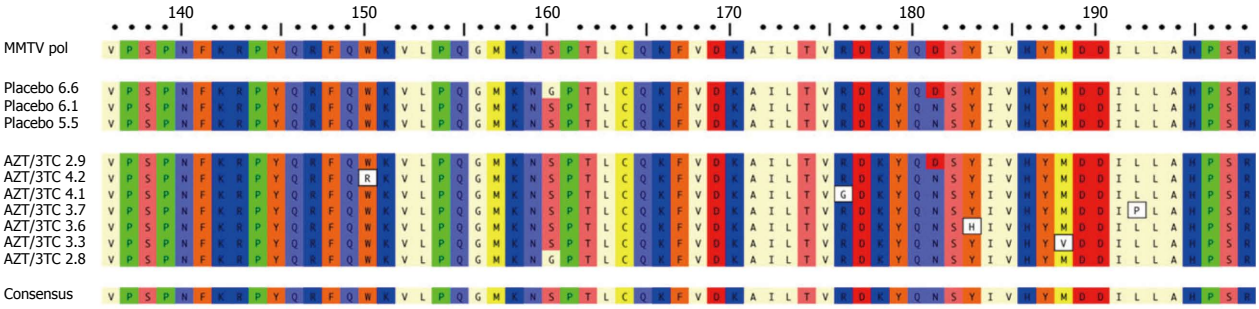

Figure 2 Variations in MMTV *pol* gene were observed after 12 wk lamivudine/zidovudine therapy in the NOD.c3c4 mouse. Alignment of amino acid sequence 136-198 of MMTV Pol P03365.2 using ClustalW alignment (MacVector 11.1 software) showing the amino acid variations W150R, R176G, Y183H, M188V and L192P in five clones derived from two mice treated with lamivudine/zidovudine that were not observed in control mice on placebo. Variants G160S and D181N were observed in mice receiving placebo and antiretroviral therapy. With permission from Sharon *et al*[8].

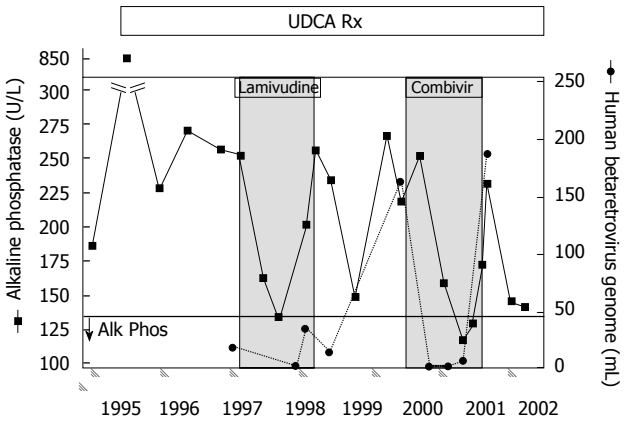

Figure 3 Viral and biochemical rebound in a patient with primary biliary cirrhosis treated with antiviral therapy. Partial biochemical response was observed after starting lamivudine treatment by 3 mo that relapsed. With the commencement of Combivir™ therapy, the patient normalized alkaline phosphatase from months 6 and 9 with a concomitant loss of viremia, which then rebounded with increased alkaline phosphatase levels and recurrent viremia. With permission from Mason *et al*[9].

transcriptase inhibitors lamivudine and zidovudine (Combivir™) alone or in combination with lopinavir boosted with ritonavir or tenofovir and emtricitabine (Truvada™) alone or in combination with Kaletra™. The important finding was that groups treated with combination antiretroviral with lopinavir - with either lamivudine/zidovudine or tenofovir/emtricitabine - developed a significant reduction in ALP, hepatic MMTV RNA levels and viral proteins within the liver (Figure 1) associated with marked amelioration in cholangitis and necroinflammatory score in the liver[8]. Whereas NOD.c3c4 mice treated with lamivudine/zidovudine or tenofovir/emtricitabine alone fared less well with hepatic biochemistry and histology. Indeed, some mice treated with lamivudine and zidovudine developed high levels of MMTV indicative of viral resistance to therapy. This occurrence was associated with variants in the MMTV *pol* gene (Figure 2), which are consistent with escape variants similarly reported with HBV and HIV infection as a result of lamivudine treatment[8]. Indeed, we observed evidence for virological resistance in our pilot studies of lamivudine and zidovudine therapy in patients with PBC, where one individual developed a picture of biochemical and virological rebound (Figure 3). In summary, the NOD.c3c4 studies suggest a role of MMTV in the development of autoimmune biliary disease and have provided a valuable resource for evaluating potential regimens in randomized controlled trials for patients with PBC.

EXPERIENCE IN TREATING PBC PATIENTS WITH ANTIRETROVIRAL THERAPY

Two major issues arise with evaluating new therapies for PBC. The first is who to treat and the second is what endpoints to use. It has been well documented that patients unresponsive to the standard of care with ursodeoxycholic acid (UDCA) have a diminished survival and are in need of additional therapy[5,44]. As multiple criteria have been used to predict response to treatment, a global PBC consortium has used collective data to demonstrate that both ALP and bilirubin act as good surrogate markers for survival and have arrived at a Global scoring system to predict patients at risk for transplantation[45-47]. These studies help to provide guidance for an accurate assessment of the patients in need of treatment with adjunctive therapy to UDCA as well as inclusion criteria for clinical trials.

The Global PBC studies also provide insight into appropriate endpoints for clinical trials. Large prospective studies have been performed to study outcomes with PBC without providing positive data leading us to rethink the appropriate endpoints for studying intervention in PBC[48,49]. For example, the methotrexate multi-center RCT of 265 PBC patients was stopped after 11 years due to lack histological effect of the treatment in 2005[49]. Subsequently, the American Association for the Study of Liver Disease (AASLD) have recommended the following for PBC studies: patients should be on UDCA (unless the medication was not tolerated), biochemical markers

with drop in ALP levels and normalization of bilirubin are satisfactory primary endpoints after 6 mo therapy and histology can be used as a primary endpoint[50]. The major discussion now is what constitutes a meaningful reduction. The criteria most recently used in a phase 3 study of obeticholic acid for patients with PBC was a reduction of ALP to less than 1.67 × the upper limit of normal and ⩾ 15% reduction and a total bilirubin less than or equal to the upper limit of normal[51].

However, these measures were not in practice for the first studies of antiretroviral therapy in patients with autoimmune liver disease. These were designed to determine whether antiviral therapy might have any biochemical or histological impact on the disease process and find out whether antiviral treatment is safe and well tolerated in patients with PBC[6,9-11].

Pilot study of lamivudine therapy

In our first study, 11 patients with PBC were treated with the reverse transcriptase inhibitor, lamivudine 150 mg daily for 1 year. The main endpoints were assessment of hepatic biochemistry and histology as determined using a modified Ishak scoring system: necroinflammatory score, bile duct injury, fibrosis stage, and ductopenia with the percentage of portal triads without bile ducts[9]. None of the patients experienced reduction of ALP below upper limit of normal and there was little change in median levels. Overall, histologic characteristics did not differ significantly between baseline and follow-up time points; if anything, there was a tendency towards worsening of scores with no reversal in ductopenia (Figure 4). Despite overall trends, some patients experienced considerable improvement in biochemical and histological parameters indicating that the treatment may have positively impacted the course of PBC. Apart from the fact that the medication was well tolerated, it was essentially a negative study.

Pilot study of lamivudine and zidovudine therapy

In our second study, 11 patients with PBC were treated with lamivudine 150 mg and zidovudine 300 mg BID for 1 year, including 7 patients who had previously participated in the lamivudine study[9]. Analyses of the biochemical data available from 10 patients revealed significant improvement in ALP after 1 year's therapy with a reduction from baseline of 218 IU/L to 150 IU/L at the end of study. The biochemical studies also showed significant reduction in ALT and AST levels at 6 mo after the initiation of antiretroviral therapy; however, this improvement was short lived and no significant changes were observed in these liver studies at 1 year compared to baseline. Paired liver biopsies were available from 7 subjects that showed an improvement in all histological parameters including the necroinflammatory score, bile duct injury, and fibrosis after 1 year of treatment (Figure 4). Overall, there was a significant improvement in ductopenia with a 30% increase in the proportion of portal tracts with demonstrable bile ducts. Considering individual patients' trends, ductopenia - defined as a finding of fewer than 80% of portal tracts with bile ducts[52] - was observed in 4 of the 7 cases at baseline. Subsequently, 6 patients demonstrated return of bile ducts with lamivudine and zidovudine treatment, whereas one patient experienced a minor reduction (Figure 4).

The reversal of ductopenia was one of the most important findings in this study. Previous investigations using UDCA therapy have reported positive impact on the lobular inflammation, piecemeal necrosis and even possible delay in the development of fibrosis/cirrhosis; however, ductopenia usually remained at the same degree in patients with a good biochemical response to UDCA and progressed in patients with an incomplete biochemical response[53-55]. A combination of UDCA plus colchicine as well as UDCA plus prednisone and azathioprine were also reported to improve an overall histological grade/score and lobular inflammation without any particular impact on the percentage of portal tracts with bile ducts[56,57]. Similarly, many immunosuppressive agents have been associated with a reduction in hepatic necroinflammatory scores but have not been shown to be effective in the reversal of ductopenia[58-62].

Randomized controlled trial using lamivudine and zidovudine

As our pilot study had delivered promising results by demonstrating substantial biochemical and histological improvements on lamivudine and zidovudine therapy, we progressed to a multicenter, double-blind, randomized placebo controlled trial[10]. Fifty-nine patients with an ALP level greater than 1.5 the upper limit of normal and stabilized on UDCA therapy were randomized to either lamivudine and zidovudine or placebo regimen for 6 mo. Liver biopsies were not reviewed due to short duration of the study and the end points were hepatic biochemistry. Patients on lamivudine and zidovudine experienced significant improvement in biochemical parameters compared to the placebo arm. They experienced a 66 IU/L reduction in ALP (21% reduction, $P < 0.04$ *vs* placebo, Figure 5). Significant differences were also achieved in the lamivudine and zidovudine arm versus placebo with serial reduction in ALT ($P < 0.03$) and AST ($P < 0.04$) levels from their baseline values, mirroring observations from the pilot study. However, the established biochemical endpoints were not reached over the 6 mo treatment period, with normalization of ALP.

This endpoint was too stringent to provide significant responses and none of the patients normalized their ALP levels over the study period. On re-examination of the study data, only a small proportion achieved responses using the newer endpoint criteria adopted

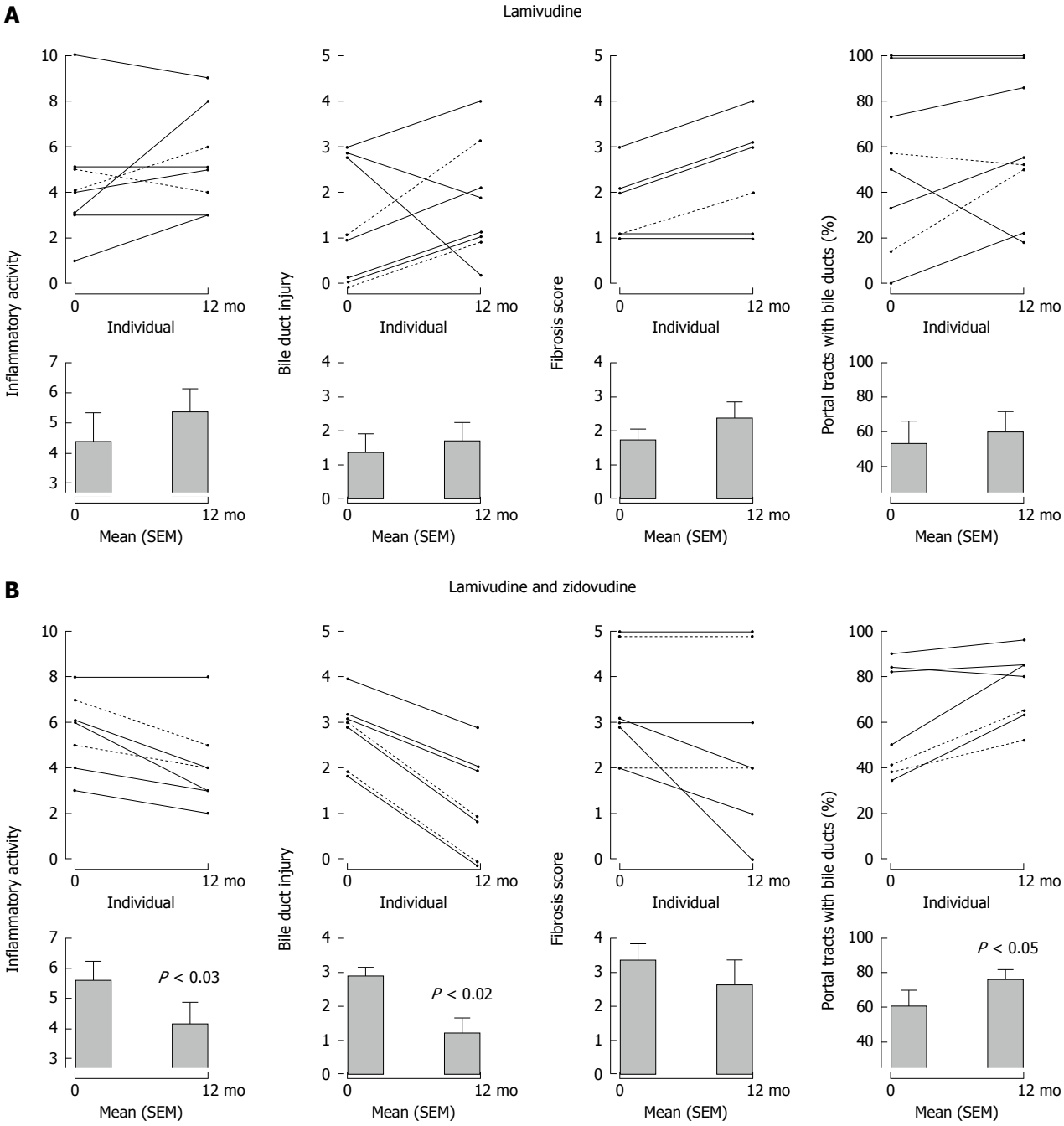

Figure 4 Serial liver biopsies assessed before and after 1 years' open label therapy with (A) lamivudine and (B) lamivudine and zidovudine showing a significant reduction in necroinflammatory activity, bile duct injury, and improvement in ductopenia for subjects receiving lamivudine and zidovudine therapy. With permission from Mason *et al*[9].

for the obeticholic acid phase Ⅲ studies[63]. However, we could only evaluate a subset of patients, as the entry criteria for the lamivudine and zidovudine study included patients with lower ALP levels of 1.5 the upper limit of normal. Moreover, several patients treated with lamivudine and zidovudine developed a clinically meaningful reduction in alkaline phosphatase at 3 mo but then developed biochemical rebound by the end of treatment suggesting viral resistance to therapy. Furthermore, patients experienced side effects with anemia and alopecia secondary to the anti-metabolic effects of zidovudine[10]. Despite the positive effects on histology in the pilot study (Figure 4), the accumulative observations from the pilot and randomized controlled trials strongly suggested that lamivudine and zidovudine alone lacked long-term efficacy for patients with PBC because of the development of viral resistance and adverse side effect profile.

Another important consideration is that lamivudine and zidovudine had little impact on limiting progressive HIV infection until the introduction of combination antiretroviral therapy with protease inhibitors[64].

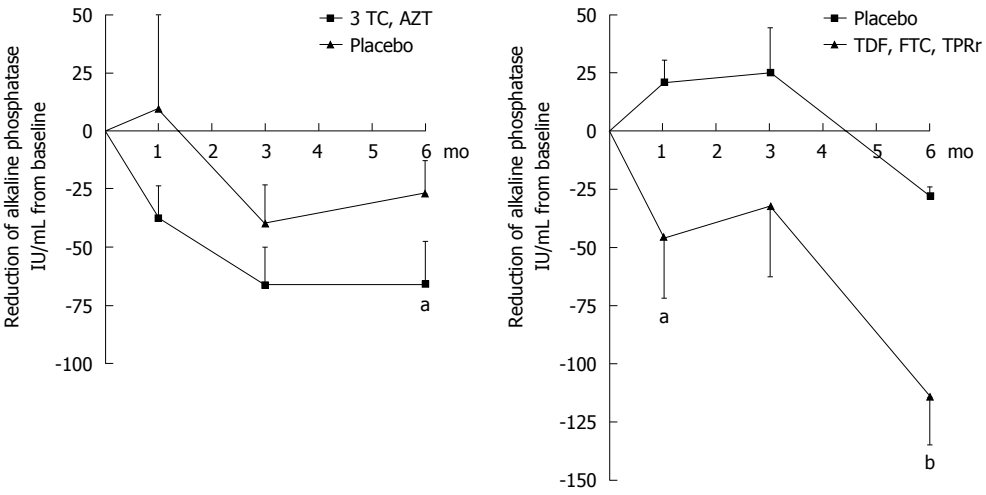

Figure 5 Incremental improvement of hepatic biochemistry observed in primary biliary cirrhosis patients maintained on UDCA receiving combination antiretroviral therapy with a protease inhibitor. Patients treated with daily lamivudine 150 mg (3TC) and zidovudine 300 mg (AZT) developed a 66 IU/mL mean reduction in ALP, whereas those receiving daily tenofovir/emtricitabine 300/200 mg (TDF, FTC) and lopinavir/ritonavir 800/200 mg (LPRr) for 6 mo (n = 13) experienced a mean ALP reduction of 114 IU/mL (two-way ANOVA, $^{b}P < 0.001$, $^{a}P < 0.05$ *vs* control). With permission, adapted from Mason *et al*[11].

Similar to observations in PBC patients, viral resistance developed within weeks of lamivudine and zidovudine therapy in patients with HIV infection. These observations prompted the consideration that combination antiretroviral therapy may be required to treat PBC patients unresponsive to UDCA. Indeed, combination antiretroviral therapy has already been reported to be efficacious in one individual with PBC. In a case report, a patient with biopsy proven PBC and evidence of co-infection with HBRV infection and HIV, was started on tenofovir, emtricitabine and lopinavir. He normalized his hepatic biochemistry studies within a year of starting combination antiretroviral therapy and importantly, he experienced a 400 IU/L reduction in ALP prior to starting UDCA[65]. Accordingly, after demonstrating the efficacy of this combination in our mouse model of PBC[8], we studied the effects of tenofovir, emtricitabine and lopinavir in a randomized controlled trial.

One last piece of the puzzle that needs to be resolved is the use of virological endpoints. The HBRV antiviral studies described so far are somewhat reminiscent of early use of interferon-α subcutaneous injection three times a week for patients with hepatitis C virus infection. Twenty five years ago, the study endpoints were normalization of hepatic biochemistry (ALT) with response rates below 20%. Whereas histological improvement was generally observed in a larger proportion of patients, the relapse rates of recurrent hepatitis C virus infection were high. Once virological endpoints were instituted with more potent regimens, we observed more robust and predictably durable antiviral responses[66]. In a similar fashion, the HBRV studies have been conducted somewhat in the dark because we have lacked sensitive virological assessment in blood. In prior studies, HBRV was generally found in plasma of 25% patients with levels at the limits of detection using real-time RT-PCR[67]. With the use of digital droplet PCR, we can now detect low level virus infection with a wide dynamic range without need for generating standard curves[68-71]. The latter employs emulsion PCR to perform individual reactions within a droplet, reducing the amount of template DNA that competes for the PCR primers. We have started to employ this method for monitoring virological response to therapy with improved detection rates.

Six month randomized controlled trial of tenofovir, emtricitabine and lopinavir

A total of 13 patients unresponsive to UDCA with serologically and histologically proven PBC were included in this randomized, placebo controlled 6 mo crossover study using tenofovir, emtricitabine and lopinavir boosted with ritonavir. We adopted similar criteria as the obeticholic acid trial for endpoints with a reduction in ALP levels to less than 1.67 × the upper limit of normal[72,73]. Following initial screening, the patients were maintained on UDCA 13-15 mg/kg and randomized into the treatment arm with tenofovir/emtricitabine 300/200 mg and lopinavir/ritonavir 800/200 mg versus placebo and then followed for a 6 mo period. Then, four patients from the placebo arm were crossed over to the treatment arm and followed for the same period.

The interim analyses in this cohort demonstrated an incremental larger reduction in ALP after 6 mo compared to prior studies using reverse transcriptase inhibitors alone (Figure 5). However by the end of the study, the reduction in ALP levels was not preserved. Only one patient (13%) cleared virus in PBMC DNA and none of the patients in the treatment arm reached

the primary biochemical endpoint at 6 mo despite the significant decline in ALP levels. The major problem was that lopinavir boosted with ritonavir was not well tolerated. First of all, it caused an increase in liver biochemistry tests in the first 3 mo. Subsequently, only a third of the patients could stay on lopinavir due to gastrointestinal intolerance throughout the whole study and this limited the enrollment into the randomized controlled trial. Indeed, the prevalence of side effects experienced by patients with PBC was over double that reported for patients with HIV[64].

Open label tenofovir, emtricitabine and lopinavir for a total of 24 mo

Once patients had completed the 6 mo trial, they were given the opportunity to continue in an open label extension study; 6 patients remained on tenofovir/emtricitabine alone, whereas the other 3 patients stayed on tenofovir/emtricitabine and lopinavir/ritonavir for the 2-year open label period. Patients on tenofovir/emtricitabine alone showed some early response but eventually did not fair any better than a historical control group of clinic patients maintained on UDCA who met entry criteria for the study. Overall, a significant reduction in ALP levels was maintained in the patients treated with combination tenofovir/emtricitabine and lopinavir/ritonavir with a continued improvement from 1 to 2 years treatment. Two of the patients (66%) cleared virus from PBMC DNA.

These studies are the first to apply antiviral therapy in patients with PBC and follow-up using a complete set of clinical, biochemical, histological and virological endpoints. The demonstrable superiority of combination therapy is encouraging despite the lack of tolerance to lopinavir. The histological and virological responses to the different treatment regimens are currently being analyzed. Liver biopsies were performed on all patients at the completion of two years therapy to determine whether long-term evolution of biochemical, clinical, and virological parameters correlate with improvement or progression in histological disease. This is deemed important because liver biochemistry can improve whereas patients develop worse histological and clinical outcomes as reported in patients with primary sclerosing cholangitis maintained on high dose UDCA therapy[74,75]. In addition, it is important to obtain histological and biochemical data over a protracted period for two years to provide important safety and tolerability data.

FUTURE DIRECTIONS FOR ANTIRETROVIRAL THERAPY IN PATIENTS WITH PBC

There is a need to find better adjunctive therapy for PBC patients unresponsive to UDCA. This cohort constitutes over a third of all PBC patients and contributes up to 10% of patients requiring liver transplantation worldwide. There are now credible data based on our long-term extension study that combination antiretroviral therapy should be studied further in the setting of randomized controlled trials as adjunctive therapy with UDCA. Some of the previous barriers to investigating antiretroviral therapy in PBC have been circumnavigated. It has been established that the majority of PBC patients have evidence of HBRV in their bile ducts and digital droplet PCR has considerably improved the frequency of detecting HBRV in peripheral blood.

While the lack of tolerability of lopinavir is disappointing, the *in vitro* studies and combination antiretroviral studies stand as a proof of principal that protease inhibitors for HIV may also serve to inhibit HBRV as well. However, HBRV was still present in all but one patient after six months therapy suggesting that despite the additional benefit of a protease inhibitor, the antiviral potency of tenofovir/emtricitabine and lopinavir/ritonavir is limited for HBRV. Ongoing laboratory studies *in vitro* and *in vivo* suggest that other HIV protease inhibitors and integrase inhibitors may provide superior antiviral effect and most of these medications are better tolerated than lopinavir/ritonavir. Once better regimens have been identified, it will be worth conducting pilot studies to assess efficacy and safety in patients with PBC, in order to provide better outcomes for those with progressive liver disease unresponsive to UDCA.

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REVIEW

Gut microbiota in autism and mood disorders

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Abstract

The hypothesis of an important role of gut microbiota in the maintenance of physiological state into the gastrointestinal (GI) system is supported by several studies that have shown a qualitative and quantitative alteration of the intestinal flora in a number of gastrointestinal and extra-gastrointestinal diseases. In the last few years, the importance of gut microbiota impairment in the etiopathogenesis of pathology such as autism, dementia and mood disorder, has been raised. The evidence of the inflammatory state alteration, highlighted in disorders such as schizophrenia, major depressive disorder and bipolar disorder, strongly recalls the microbiota alteration, highly suggesting an important role of the alteration of GI system also in neuropsychiatric disorders. Up to now, available evidences display that the impairment of gut microbiota plays a key role in the development of autism and mood disorders. The application of therapeutic modulators of gut microbiota to autism and mood disorders has been experienced only in experimental settings to date, with few but promising results. A deeper assessment of the role of gut microbiota in the development of autism spectrum disorder (ASD), as well as the advancement of the therapeutic armamentarium for the modulation of gut microbiota is warranted for a better management of ASD and mood disorders.

Key words: Gut microbiota; Mood disorders; Autism; Depression; Gut microbiota modulation; Fecal microbiota transplantation

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Core tip: Up to now, available evidences display that the impairment of gut microbiota plays a key role in the development of autism and mood disorders.

The application of therapeutic modulators of gut microbiota to autism and mood disorders has been experienced only in experimental settings to date, with few but promising results, that suggest the microbiota modulation as a therapeutic approach for autism and mood disorders.

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INTRODUCTION

The gut microbiota, composed of thousands of different microbial species and more than 15000 kinds of bacteria for a weight equal to 1 kg, represents the first protection system of the gastrointestinal (GI) apparatus. The presence of the microbiota varies within the gastrointestinal tract, from few microorganisms in the stomach and small intestine, up to a concentration of approximately 1.012 bacteria in the colon, mostly represented by the *Firmicutes* and *Bacteriodetes phyla*[1,2]. Within the species that compose the microbiota, it's also possible to recognize the kingdom of *Archaea* and *eukaryotes*, and many viruses and *bacteriophages*[3,4]. Finally, there were several families of fungi, whose physiological role in the gastrointestinal system is still unclear.

The functions performed by the flora are manifold; in addition to the contribution to the establishment of the intestinal barrier, it promotes its maintenance, stimulating epithelial regeneration through the production of short chain fatty acids (SCFAs), leading to mucus production and exerting a trophic action on the mucous membrane[5].

The gut microbiota is involved in the maturation of the immune system: it stimulates innate immunity in the early years of life, leading to the maturation of the GALT, and acquired immunity, through stimulation of local and systemic immune responses[6]. Known, finally, is the role in the synthesis and metabolism of certain nutrients, hormones and vitamins, and clearance of drugs and toxic.

The human body, completely sterile at birth, is immediately in contact with a large amount of microbial communities, including the fecal, vaginal and skin microbiota of the mother. Subsequently, the composition of the flora undergoes changes, influenced by age, sex, state of immune maturation and by environmental factors.

The flora acquires its stability between 6 and 36 mo of life; in that period it's already possible to distinguish between a constant endogenous flora (core microbiota) and a still provisional one, highly sensitive to external stimuli[7,8].

In physiological conditions, the continuous stimulation of the immune system by the gut microbiota leads to a state of "low-grade physiological inflammation", which is a rapid and effective mechanism of defence against pathogens[9]. In addition, the flora exerts its protective role competitively, metabolizing those nutrients needed for pathogens survival, and producing molecules that inhibit the growth of such microbes[10].

Sonnenburg *et al*[11] has shown that the introduction of a compound of *Bacteroides thetaiotamicron* and *Eubacterium rectale* is able to induce the production of particular mucosal glycans, which may be metabolized exclusively by these bacterial species and not by pathogens, thus preventing their proliferation.

The hypothesis of an important role of gut microbiota in the maintenance of physiological state into the GI system is supported by several studies that have shown a qualitative and quantitative alteration of the intestinal flora in a number of gastrointestinal and extra-gastrointestinal diseases.

GUT MICROBIOTA AND PSYCHIATRIC DISORDERS: A FOCUS ON AUTISM SPECTRUM DISORDER AND MOOD DISORDERS

Recent data show the strong correlation between dysbiosis and conditions such as obesity, allergies, autoimmune disorders, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and psychiatric disorders[12-16].

Due to these new evidences about the fundamental role of gut microbiota in the alteration of immune, neural, and endocrine pathways, the so-called "gut-brain axis" is acquiring new significance, even if the communication routes are not still defined[16-18].

At the beginning of the past century, first hypotheses aroused about the correlation between these two systems; probably the most practice evidence can be found in a work of an army surgeon, who noted the correlation between a patient's gut function and his mood, monitoring gastric secretions through a fistula in his stomach[13].

In the last few years, much research has been done in this direction, underlying the importance of dysbiosis in the etiopathogenesis of pathology such as autism, dementia and mood disorder. The evidence of the inflammatory state alteration, highlighted in disorders such as schizophrenia, major depressive disorder and bipolar disorder[19-23], strongly recalls the microbiota alteration and highly suggests an important role of the alteration of GI system also in neuropsychiatric disorders.

In particular, the dysbiosis and the consequent alteration of intestinal permeability lead respectively to the production and spread into the bloodstream of a potent pro-inflammatory endotoxine, namely

lipopolysaccharide (LPS). This small molecule has an important influence in the modulation of the central nervous system (CNS), increasing the activity of areas deputed to the emotionalism control such as amygdala[24]. It also lead to production of inflammatory cytokines that alter the physiological brain activity, modulating the neuropeptides synthesis[25].

Rhee *et al*[26] highlighted the importance of bidirectional connections between gut and brain that occurs in both healthy and diseased states focusing attention on enterochromaffin cells. The signals generated by the stimulation of these pathways due to intraluminal gut stimuli, running on nervous system, strongly modulate the brain activity, including pain perception, immune-response modulation, emotional control and many other homeostatic functions.

However, this influence is not unidirectional, but is a continuous communication: the CNS is able to change the composition of microbiota and to alter the equilibrium in the gut permeability, modulating motility and secretion through the activation of the hypothalamus pituitary-adrenal (HPA) axis, autonomic and neuroendocrine system with an immediate impact on gut microbiota[26,27]. In this regard O'Mahony *et al*[28], showed that early maternal separation in rats increased corticosterone systemic level, resulting in the alteration of immune response and fecal microbiota. Among several actors of this axis, important molecules have been studied such as vasoactive intestinal peptide (VIP) serotonin, melatonin, gamma-aminobutyric acid (GABA), catecholamines, histamine and acetylcholine[29-32], even if interaction way and acting routes of these molecules is not fully established.

Autism spectrum disorder (ASD) is a range of developmental neuro-behavioral disorders characterized by restricted and repetitive behaviour, impaired social interaction and communication; among these, autism represents the primary type of ASD[12,16].

The possible role of gut microorganism in the pathogenesis of such disorders has been widely deepened by several studies in animal models using different approaches: comparison of gut microbiota composition between affected samples an controls; observation of behaviour changes after administration of gut microbiota modulators in affected subjects rather than virulence factors in controls.

It has been demonstrated that a large amount of species under the *Clostridium* genus (10 times more) characterised the qualitative composition of fecal samples of autistic children[33-35]. Then, the composition of microbiota has been characterized, showing an imbalance of *Bacteroidetes* and *Firmicutes phyla,* with an increased presence of *Bacteroidetes* and other gut commensal such as *Bifidobacterium*, *Lactobacillus*, *Sutterella*, *Prevotella, Ruminococcus genera* and *Alcaligenaceae* family[36-40].

In the 1998, Bolte[41] observed that a significant percentage of individuals with autism had a history of extensive antibiotic use that significantly disrupt protective intestinal microbiota. On this basis, he outlined the possibility of a subacute, chronic tetanus infection of the intestinal tract that underlies the pathogenesis of symptoms in autism observed in some individuals.

Sandler *et al*[42] speculated that the alteration of autochthonous gut flora microbiota leads to the colonization by bacteria able to produce neurotoxins, contributing, at least in part, to their autistic symptomatology. On this basis, they treated a small group of children affected by regressive-onset autism with poor oral absorption-antibiotic. At the end of treatment, short-term improvement was noted using multiple pre- and post-therapy evaluations.

It has been also studied the consequences of gut barrier alteration contribute to ASD. A study carried on by Emanuele *et al*[43] showed that LPS serum levels were significantly higher in autistic patients compared to heath individuals and correlated with socialization scores in an inverse and independent manner. These evidences support a role of microbiota and, generally, of an alteration of the gut barrier in its integrity, in the genesis of ASD.

Nevertheless, the existence of a gastrointestinal dysbiosis as an actor in the ASD etiopathogenesis remains a controversial topic. In this regard, the study carried on by Gondalia *et al*[44] didn't showed clinically meaningful differences in the gut microbiota characterization between children affected by autism and their neurotypical siblings.

Depression is a major form of mood disorder characterized by depressed mood and/or recurrent thoughts of death and/or loss of interest or pleasure in life activities present over a period of at least 2 wk, accompanied by at least five additional symptoms that cause clinically significant impairment in social, work, or other important areas of functioning[13]. It results from neuro-psychiatric disturbance, immunological deregulation, genetic factors and environmental influences; nevertheless, a correlation with gut microbiota is emerging[45-47]; Through humoral route, microbiota can also influence CNS neurotransmission: it has been demonstrated that in GF mice anxiety-like behavior is reduced and modulated after restoration of the intestinal microbiota[48-50]. In particular, administration of *Lactobacillus* sp, *Bifidobacteria* sp, *L. helvetucys*, *B. longum*, *L. rhamnosus* and *Lactobacillus farciminis* in murine sample lay to an improvement of depression and anxiety symptoms[51].

In particular, an alteration of intestinal permeability, causing high level of LPS into the bloodstream, lead to the activation of inflammatory and immune response; these processes have been hypothesized as causative factors in psychiatric disorders such as depression[52,53]. Moreover, as support to this hypothesis, it has been demonstrated that the administration of LPS in healthy subject is associated to increase of pro-inflammatory cytokines and plasma norepinephrine, whit higher

Table 1 Alterations of gut microbiota found in autism and mood disorders

Disease	Microbiota alterations
Autism	Imbalance of *Bacteroidetes/Firmicutes* ratio Increase of *Bacteroidetes* phylum, *Bifidobacterium*, *Lactobacillus*, *Sutterella*, *Prevotella*, *Ruminococcus* genera, *Alcaligenaceae* family
Depression	Increase of *Alistipes* Negative correlation between *Faecalibacterium* abundance and severity of disease

depression rates[54].

Among clinical studies conducted, gut microbiota has been characterized, showing an overexpression of *Alistipes* in patients affected by depression disorder[47]. The overexpression of this bacterium, a genus in the phylum of *Bacteroidetes,* has been demonstrated in other disorders, such as chronic fatigue syndrome and in IBS[55,56]. This evidence lead to speculate about a gut microbiota alteration as common mechanism of action in the genesis of these disorders. Moreover, *Alistipes* has been linked to depression mood by generation of inflammatory molecules able to spread into the bloodstream in condition of altered intestinal permeability[47,51,57]. Another study, carried on by Jiang *et al*[58], confirmed the overexpression of *Alistipes* in psychiatric disorder and observed a negative correlation between expression of *Faecalibacterium* and the severity of depressive manifestations. An overview of main alterations of gut microbiota in autism and depression is available in Table 1.

POTENTIAL FOR THERAPEUTICS

Antibiotics

Antibiotics are the oldest drugs used in the management of diseases of the gastrointestinal tract. Their use, especially for infectious diseases, can achieve an alteration of the composition of the gut microbiota that can lead to significant side effects, not the least of antibiotic-associated diarrhoea due to *Clostridium difficile*[59]. Despite this, the antibiotic therapy is currently encouraged in the management of disorders such as IBS, IBD and SIBO in which the modulation of the intestinal flora leading to a net clinical improvement.

Currently, researches are being made in order to clarify the modulation of gut microbiota in the management of psychiatric disorder. It has been demonstrated that reduction of luminal LPS concentration due to antibiotic therapy lead to attenuation of HPA axis stress response and to increase of hypothalamic pro-inflammatory cytokines expression[60].

Desbonnet *et al*[61] have reproduced the effect of microbiota depletion on murine specimens: they administered them a combination of antibiotics and then assessed the effects from weaning onwards on adult cognitive, social and emotional behaviours and markers of gut-brain axis dysfunction in mice. They demonstrated that the reduction and diversity of the gut microbiota influences adult behaviours and key neuromodulators of the gut-brain axis: it reduced anxiety, induced cognitive deficits, altered the brain hormone expression and altered dynamics of the tryptophan metabolic pathway.

In support of these findings, some studies have successfully tested minocycline, second-generation tetracycline, as a treatment for depression, on the basis of its neuroprotective activities and regulation of pro-inflammatory agents[62,63].

In an other study, 11 children affected by ASD have been treated with vancomycin: after the planned 8 wk of treatment, communication and behaviour tests improvement has been observed[42].

Thus, it's possible to speculate that antibiotic treatment, through modulation of gut micriobiota, should be able to influences symptoms and expression of psychiatric disorders.

Probiotics

Probiotics are defined as live micro- organisms, preferentially of human origin, that upon ingestion in specific and sufficient numbers confer non-specific health benefits to the host[64].

Currently widely used in gastrointestinal system disorders, they exert their therapeutic effect by interacting on various levels in the reconstitution of the gastrointestinal barrier. In addition to a direct effect in the composition of Gut Microbiota, they are able to modulate the GI barrier through the increase of mucin production by globet cells, strengthening the tight junctions and thus the apical intercellular adhesion[65-68].

Probiotics are also involved in the modulation of the immune and inflammatory response by promoting the production of regulatory T cells. They may also regulate the Th1 response, by inhibition the production by the dendritic cells of pro-inflammatory cytokines such as IL-12, TNF-α and INF-α, or increase the expression of anti-inflammatory mediators such as IL-10 and β-TFGF[67].

Some studies tested probiotics as symptoms' modulator in disorders such as anxiety and depression. For example, Bravo *et al*[69] demonstrated that chronic administration of *L. rhamnosus* modulates GABA expression in CNS in rat, leading to a reduction in the hippocampus, amygdala, and locus coeruleus and to an increase in cortical regions. Furthermore, it reduces levels of corticosterone induced by stress and depression- and anxiety-related symptoms. In particular, these events didn't appear in vagotomised mice, indicating a fundamental role of vagal sings, and generally of neuronal transmission, in the gut-brain axis.

Similarly, combination of *L. helveticus* and *B. longum* appears to have an anxiolytic-like activity in

rats and, in addition to a diet formulation containing high levels of polyunsaturated fatty acids (PUFAs) n-3, to reduce post-MI depression[70,71].

Despite these impressive results, few clinical trials have been conducted with poor results. A double-blind, randomized clinical trial demonstrated that the daily administration of mixture of probiotics containing *L. helveticus* and *B. longum* for a month reduce psychological distress in healthy controls[70].

Rao *et al*[72] showed that the daily administration of *Lactobacillus casei* for two months improves anxiety related symptoms in subject affected by chronic fatigue syndrome[58].

However, the daily assumption of *L. casei* enriched milk didn't show significant effects in term of mood in healthy individuals while seemed to have potentially negative effects on recall memory[73].

Finally, Hsiao *et al*[74] showed that the oral administration of *Bacteroides fragilis*, improved some mood symptoms- such as anxiety, stereotypical behaviour and sensorimotor gating-in a maternal immune activation (MIA) animal model that is known to display features of ASD.

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) represents the injection of filtrate stools from a healthy donor to a patient for the healing of a specific disease. Despite it had been sporadically used in ancient times, its first application in contemporary medicine in English literature, dates back 1958, when Ben Eiseman infused fecal material in four patients with pseudomembranous colitis[75]. After this pioneering experience, several attempts were reported over time for the treatment of *C. difficile* infection. To date, several systematic reviews and meta-analyses[76-78], as well as three randomized controlled trials[79-81], outlined the undoubted efficacy of FMT for the treatment of recurrent *C. difficile* infection. Some proof-of-concept randomized controlled trials investigated the efficacy of FMT in metabolic syndrome[82] and IBD, respectively[83,84]. In particular, it has been reported that FMT improved sexual function in patients with Crohn's diseases: this finding might get stronger the connection between of gut microbiota and depression/mood[85]. At present, despite the theoretical background for the application of FMT to autism is sound, to date it was experienced only in two autistic children, in whom it showed an amelioration of specific symptoms[86].

CONCLUSION

In the last few years, the importance of gut microbiota in the maintenance of physiological state into the GI system is supported by several studies that have shown a qualitative and quantitative alteration of the intestinal flora in a number of gastrointestinal and extra-gastrointestinal diseases. The application of gut microbiota modulators, such as probiotics, antibiotics, up to FMT, has been widely experimented as therapeutic instrument for GI diseases with exciting results.

Up to now, available evidences display that the impairment of gut microbiota plays a key role also in the development of autism and mood disorders, but the mechanism through which it does is not fully clear. The application of therapeutic modulators of gut microbiota to autism and mood disorders has been experienced only in experimental settings to date, with few but promising results.

A deeper assessment of the role of intestinal flora in the genesis and development of mood disorders and ASD is currently required; the knowledge advancement of the modulation of the intestinal flora not only about possible modalities but also about the timing in which this should be done, would lead to a new and safe therapeutic weapon in the management of ASD and mood disorders.

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REVIEW

Capsule endoscopy: The road ahead

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Abstract

Since its introduction into clinical practice 15 years ago, capsule endoscopy (CE) has become the first-line investigation procedure in some small bowel pathologies, and more recently, dedicated esophageal and colon CE have expanded the fields of application to include the upper and lower gastrointestinal disorders. During this time, CE has become increasingly popular among gastroenterologists, with more than 2 million capsule examinations performed worldwide, and nearly 3000 PubMed-listed studies on its different aspects published. This huge interest in CE may be explained by its non-invasive nature, patient comfort, safety, and access to anatomical regions unattainable *via* conventional endoscopy. However, CE has several limitations which impede its wider clinical applications, including the lack of therapeutic capabilities, inability to obtain biopsies and control its locomotion. Several research groups are currently working to overcome these limitations, while novel devices able to control capsule movement, obtain high quality images, insufflate the gut lumen, perform chromoendoscopy, biopsy of suspect lesions, or even deliver targeted drugs directly to specific sites are under development. Overlooking current limitations, especially as some of them have already been successfully surmounted, and based on the tremendous progress in technology, it is expected that, by the end of next 15 years, CE able to perform both diagnostic and therapeutic procedures will remain the major form of digestive endoscopy. This review summarizes the literature that prognosticates about the future developments of CE.

Key words: Capsule endoscopy; Biopsy; Drug delivery systems; Capsule endoscope locomotion; Capsule localization

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Core tip: Since its introduction into clinical practice 15 years ago, small bowel capsule endoscopy (CE) has

revolutionized direct endoscopic imaging of the gut. During this time, CE has gained tremendous popularity among gastroenterologists, and a vast research pertaining to its different aspects has been published. Dedicated esophageal and colon CE have expanded the field of application to upper and lower gastrointestinal disorders. However, besides its recognized advantages, CE also has several limitations such as the lack of therapeutic capabilities, the inability to obtain biopsies and control its locomotion. Active research is in progress to overcome the current limitations, while the latest advances in CE technology enable us to look forward to a next generation CE capable of performing both diagnostic and therapeutic procedures. This review summarizes the literature that prognosticates about the future of CE.

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INTRODUCTION

Fifteen years have passed since small bowel capsule endoscopy (CE) was launched[1], revolutionizing noninvasive direct visualization of the small bowel, considered until then the "black box" of the gastrointestinal (GI) tract. During this time, CE has been used extensively, with more than 2 million capsules swallowed worldwide[2] and nearly 3000 PubMed-listed studies pertaining to its different aspects published[3]. Technical progress led to the introduction of some updated versions (2nd and 3rd generations) of CE for the small bowel and the manufacturing of the CE designed for esophagus and colon. In just a few years, CE has evolved very rapidly, becoming an invaluable tool for examination of almost the entire GI tract, and its diagnostic achievements have by far exceeded early expectations. Still, CE is not an ideal tool, as it has several limitations, including the lack of therapeutic capabilities, inability to control its locomotion and thus, to revisualize critical areas and obtain biopsies. The objective of many research groups worldwide is to overcome these limitations and develop a new generation of CE with higher diagnostic yield and therapeutic capabilities. Of course, it is very difficult to predict the future in medicine, and would be for CE. However, based on the extraordinary developments seen in just 15 years since its emergence, and the tremendous progresses of modern technology, it can be anticipated that, by the end of next 15 years, the new generation of CE able to perform both diagnostic and therapeutic procedures in a noninvasive, painless, and elegant manner will remain the major form of digestive endoscopy, covering the entire GI tract from mouth to anus, as its inventors have dreamed. This review summarizes available literature that prognosticates about the future developments in CE.

BRIEF LOOK BACK AND THE CURRENT STATUS

The first model of CE called M2A (meaning "mouth to anus") was launched in 2000 by Given Diagnostic Imaging, Yoqneam, Israel[4], and the merits for its design belonged, in a similar degree, to the Israeli engineer Gavriel Iddan and the British gastroenterologist Paul Swain[1,4]. A year later, M2A was approved for clinical use in Europe and the United States, and after the advent of esophageal CE, M2A changed its name into PillCam SB (meaning "small bowel"). Several other companies have also developed small bowel endoscopic capsules: EndoCapsule (Olympus Corp., Tokyo, Japan)[5], OMOM capsule (Jinshan Science and Technology Company, Chongqing, China)[6], Mirocam (IntroMedic Co., Seoul, South Korea)[7], and CapsoCam SV1 (CapsoVision, Saratoga, CA, United States)[8], all having many similar characteristics and diagnostic performances to PillCam SB, but differing with regard to image acquisition rate, field of view, battery life, dimensions, and technology for transmission of images. Given Imaging has also developed PillCam ESO and PillCam COLON for the evaluation of esophageal and colonic diseases, respectively[9,10]. Improvements in technology have led to the development of 2nd and 3rd generation CEs which overcome some limitations of the 1st generation CE by increasing the view angle, extending the effective battery life, and including several others systems which offer superior image quality, tissue coverage, and interpretation efficiency[11-13].

In only 15 years since the introduction of CE into clinical practice, its achievements have exceeded what was previously thought as possible. Thus, CE has revolutionized the evaluation of obscure gastrointestinal bleeding (OGIB) and unexplained iron deficiency anemia (IDA)[13-15], becoming the first-line modality in the diagnosis of both. The role of CE in OGIB/IDA is supported mainly by its diagnostic performance, which is superior to other diagnostic modalities (push enteroscopy, intraoperative enteroscopy, small bowel barium radiography, CT-enterography, CT-angiography, MR-enterography), as well as by its positive impact on patient management and outcome[14,16-21]. When CE was compared to double-balloon enteroscopy, a similar diagnostic accuracy for OGIB was reported[22]. CE examination leads to therapeutic endoscopic or surgical interventions and, consequently, to bleeding being stopped and outcomes improved[23,24].

Thanks to its capacity to directly visualize mucosa of the entire small bowel, CE has undoubtedly contributed substantially to progress in diagnosis, therapeutic

decision, and outcome in Crohn's disease (CD). Reviews of existent literature on CE diagnostic yield, for both suspected and known small bowel CD, show it to be superior to other diagnostic techniques such as small bowel follow-through, enteroclysis, push-enteroscopy, ileo-colonoscopy, and CT-enterography[25-27]. CE is superior to MR-enterography in identifying small bowel mucosal lesions, while MR-enterography is superior to CE in diagnosing mural and extra-enteric lesions[28]. In patients with known CD, an important treatment goal is mucosal healing which can be reliably assessed by CE[29-31].

CE has an 8-fold magnification capacity and a minimum size of lesion detection of 0.1-0.2 mm, so that villi can be easily observed during a procedure; therefore, it may be a useful noninvasive diagnostic tool in patients with suspected or established celiac disease[32,33]. However, CE is actually an alternative to endoscopy with biopsy only in patients clinically suspected of celiac disease unable or unwilling to undergo conventional endoscopy.

CE has become the procedure of choice for detecting small bowel polyps in hereditary polyposis syndromes like Peutz-Jegher syndrome and familial adenomatous polyposis[34,35]. In addition, widespread use of CE has more than doubled the diagnosis rate in small bowel tumors[36-41].

Esophageal capsule endoscopy, although at 3rd generation, has limited role in clinical practice and it is still under evaluation[42]. Colon capsule is also under evaluation, and is currently recommended in case of incomplete colonoscopy and in patients unwilling or unable to perform colonoscopy[43,44].

Limitations of current capsule endoscopy

Although CE has seen tremendous advances in a short period of time since its introduction in clinical practice, it has several limitations. Thus, CE remains a purely visual technique with no ability to obtain biopsy specimens or perform therapeutic maneuvers. The most obvious drawback is the operator's inability to control its locomotion through GI tract. The capsules presently on the market are unable to localize or mark the location of detected lesions. Visualization may be impaired by the presence of food materials or bubbles and, in contrast with conventional endoscopy, CE cannot perform flushing, suctioning, or air insufflation to obtain better images. All capsules for clinical use are powered by limited-life batteries which may be depleted before the examination is complete. The rate of missed lesions is still high for those located in the duodenum and proximal jejunum, where the transit is more rapid than in the distal segment of the small bowel. Reading time for interpretation is another shortcoming of CE, as it takes more than 1 h to read a full 8-h examination. Finally, the costs are still high.

FUTURE EXPECTATIONS IN CAPSULE ENDOSCOPY

The future of CE is difficult to predict ("Prediction is very difficult, especially about the future" - Niels Bohr, Nobel Prize winner, 1885-1962), although novel technologies may lead to developments which today seem almost unimaginable. Improvements achieved in just 15 years since the introduction of CE in clinical practice go beyond what was previously thought as possible. GI endoscopy has had a similar history: initially limited only to viewing the esophagus/gastric lumen, it has improved progressively over a few decades, developing into an accurate diagnostic and therapeutic technique. CE also started as a tool for visualizing only the "black box" (small bowel) which has long been the final frontier of the GI endoscopy, and it evolved very rapidly to become a non-invasive endoscopic tool in the examination of almost the entire GI tract.

Most likely, over the next 15 years, CE will slowly replace diagnostic standard endoscopy and take over most therapeutic procedures with no pain and no need for sedation. We know that several research groups throughout the world are working to develop new multifunctional capsules with diagnostic and therapeutic capabilities extending far beyond our imagination. What we do not yet know is whether the future CE will be "universal", containing both diagnostic and therapeutic modules (an "ideal" CE)[45] or "specific", for diagnosis or therapy[11].

Maneuverable capsules

In contrast to standard endoscopy, the movement of the current capsule endoscopes through the GI tract is passive, ensured by peristaltic motion, the operator being unable to control the endoscopic navigation (right and left, back and forth) in a given area. It is of the upmost importance to solving the CE's maneuvering limitation so as to increase its diagnostic yield and allow targeted biopsy and even drug delivery. Besides enhancing diagnostic yield, a capsule whose locomotion can be controlled will reduce the amount of energy consumed, examination time, as well as the rate of capsule retention. Even more, an active control of the endoscopic capsule would allow us to examine the stomach, and finally, the entire GI tract[46].

Systems that can be used to propel or steer the capsule are under development. There are two locomotion systems: an internal one, integrated on-board the capsule, and an external one (outside the capsule), most frequently based on magnetic fields. Some proposed internal systems consist of legged-like mechanisms (propellers/paddles) that can be deployed by the capsule to resist peristaltic movements, while the external locomotion systems usually use a capsule

covered with a magnetic shield which can interact with external magnetic fields created by an electromagnet or permanent magnet. Electromagnets require bulkier equipment by comparison to permanent magnets[47-50].

The legged-like device approach consists of providing the capsule with propellers/paddles which will start functioning on demand during capsule navigation through various segments of the GI tract. A four-legged capsule, two in the front and two in the rear, has been proposed, an eight-legged capsule was also suggested to be feasible, and even a twelve-legged locomotion capsule was designed to improve propulsion and reduce tissue injury[47,51-53]. However, several technical drawbacks such as insufficient space available within the capsule and high power consumption should be overcome. In addition, a failure in the synchronization of the legs may cause damage to the GI tissue.

Magnetic control appears to be the most attractive and promising approach. It is based on the principle that a large external magnetic field created by a permanent magnet or electromagnet near the patient interacts with a small internal magnet component integrated into the capsule to provide an active control of the endoscopic capsule[48]. Given Imaging has incorporated a magnet inside one of the domes of a standard PillCam colon capsule, which can be manipulated with an external handheld magnet moved on the patient's abdomen[54,55]. Using such magnetically maneuverable capsule, one study reported > 75% of gastric mucosa visualized and no adverse events[55]. Siemens (Siemens Medical, Erlangen, Germany) and Olympus (Olympus America, Center Valley, PA, United States) have recently tested the prototype of a magnetically guided capsule endoscope that uses a three-dimensional, external magnetic field which interacts with the magnet inside the capsule, allowing the capsule to be moved forward or backward[56,57]. Rey *et al*[58,59] made the first blinded comparative clinical trial on gastric examination in humans, comparing a magnetically guided capsule endoscope with a conventional high-definition gastroscope, and found a similar diagnostic yield for both methods. Rahman *et al*[60], using the Intromedic MiroCam-Navi system, reported a high degree of visualization, control, and maneuverability with this system. A robotic magnetic navigation system used in cardiology (Niobe, Stereotaxis Inc., St. Louis, MO, United States) has been suggested for CE but has been tested only in plastic phantoms[61]. Several other versions of endoscopic capsules magnetically propelled by a robotic arm have been proposed[62].

Two research projects funded by the European Union aim to develop a self-propelling minirobot pill. One is *VECTOR* (Versatile Endoscopic Capsule for gastrointestinal Tumor Recognition and Therapy) for early diagnosis and treatment of GI cancer[63], and the other is *NEMO* (Nano-based capsule Endoscopy with Molecular imaging and Optical biopsy) which designed to combine optical, nano, and maneuvering technologies in a new capsule with different diagnostic and therapeutic capabilities[64].

A videocapsule endoscope called Compact Photonic Explorer (CPE), measuring 5 mm in size, has been developed at the City University and City College of New York. It can be manipulated externally by remote controlled radio signal and may be used in the future for diagnostic and therapeutic means[65]. Recently, a mathematical model of an electrically propelled capsule endoscopy has been proposed, using double pairs of electrodes, and which is able to move the capsule forward and backward at a speed of 2.91 mm/s and 2.23 mm/s, respectively[66].

To summarize, the development of propelled/steerable capsules will represent a major advance of capsule technology, which will open a myriad of possibilities, including a more detailed evaluation of affected areas and prelevation of biopsy specimens, endoscopic targeted therapy, examination of the stomach, thus the entire GI tract becoming virtually as accessible as the skin[67]!

Biopsy

Once a maneuverable capsule is developed, the next step is to obtain a tissue sample. Several biopsy devices have been developed and used on animal models. A spring-loaded device similar to the Crosby capsule, guided by real-time imaging and RF-controlled remote manipulation, and a capsule using Micro-Electro-Mechanical-Systems (MEMS) technology have been successfully tested[68]. Both NEMO and VECTOR projects develop capsules designed for virtual biopsies and drug delivery[63,64]. The rotational Micro Biopsy Capsule Device (Seoul, South Korea) which contains a triggering part with paraffin block and a rotational tissue-cutting razor (biopsy part) has been tested[69]. A tethered capsule endomicroscopy of the esophagus, which uses optical frequency domain imaging technology and enables 3D imaging of esophagus in microscopic detail, has also been developed[70]. This capsule endomicroscope is able to differentiate Barrett's esophagus from normal esophageal mucosa. Other magnetic capsules using untethered microgrippers to grab tissue samples or magnetic torsion spring mechanism have been designed[71,72].

Optical enhancing techniques could lead to optical "biopsy", which refers to a method of obtaining a morphological diagnosis without biopsy specimens, and prototype endoscopic capsules with such technology have been developed, including the wireless spectroscopic compact photonic explorer for diagnostic optical imaging to detect microscopic malignancy[65]. One research group integrated near-infrared fluorescent probe in CE to enhance optical diagnosis of neoplasia, which proved able to distinguish adenomatous tissue in

experimental colitis in mice[73].

Power source

At present, available endoscopic capsules use two coin-shaped, silver-oxide batteries that can generate 20 mW of energy, far too little to accomplish the multiple diagnostic and therapeutic tasks of the future capsule, most of them requiring power consumption. In addition, batteries occupy most of the space in an endoscopic capsule. Therefore, increased power supply and reduced size of batteries, to leave enough space to incorporate diagnostic/therapeutic components into the capsule, are essential for further developments in CE technology. A solution may be lithium ion microbattery technology which could provide a power density up to 2000-times higher than other microbatteries[74]. Recently, Rathore *et al*[75] using Ultrascale FinFET 16 nm technology for manufacturing endoscopic capsules (instead of 18 μm used for conventional endoscopic capsules) have reported an increased battery life, reduced power consumption with up to 50%, and a reduced size of the capsule by 12% compared to traditional capsules. An alternative method to reduce battery consumption is to use low complexity video compression technology that saves radiofrequency (RF) transmitting power[76].

External rechargeable batteries (from an extra-corporeal power supply) using RF, microwave or electric induction, and even "battery free" CE using wireless power transmission (WPT) technology are created. An excellent overview of the development of emergency WPT technique for application in CE has been recently published by Basar *et al*[77]. WPT system employs a transmitting coil positioned outside the human body and a receiving coil installed within the CE, thereby eliminating the need for an internal battery[78]. Thus, the RF System Lab (Nagano, Japan) was the first to use WPT technology in their Sayaka and Norika capsules[79], and several publications centered on WPT technology for the endoscopic capsule[77,80]. Jia *et al*[80], using WPT technology, have reported on its ability to transmit 500 mW of electricity, which is significantly higher than the amount generated by current batteries used for the endoscopic capsules available on the market.

An alternative solution will be the development of three-dimensional microbattery technology for geometrical energy and power density of battery[81,82], and many research groups are working in the field, still progressing in several laboratories.

Targeted drug delivery

Unfortunately, none of the current capsules is able to perform therapy. New capsule devices are under development in order to enable drug delivery in specific diseased areas of the GI tract. A number of clinical situations can benefit from targeted drug delivery such as the use of hemostatic spray to an active bleeding lesion or localized application of steroid/immunomodulation for CD. One capsule prototype is able to deliver an injection of 1 mL of targeted medication while using a holding mechanism[83]. To achieve this, an accurate control mechanism of capsule positioning and a drug release mechanism should be incorporated into a capsule endoscope. As future capsules will most likely be smaller, space limitation within the capsule is an important impediment when incorporating such mechanisms[84].

Philips company (Philips Research, Eindhoven, The Netherlands) has launched an "intelligent" pill (iPill) measuring 11 mm × 26 mm and incorporating a microprocessor, battery, pH sensor, temperature sensor, radiofrequency transceiver, fluid pump, and a drug reservoir[85]. Tracking of the iPill in the GI tract is based on information regarding pH change and gut transit time. Once tracked in the aimed area, the iPill will open and deliver the drug under the control of the microprocessor. The iPill is under trial in CD and colorectal cancer[68,85,86].

Several other wireless capsules, such as the Gastro-target telemetric capsule (Gastrotarget, Tonawanda, NY, United States), High-frequency capsule (Battelle-Institute V, Frankfurt, Germany), Telemetric capsule (INSERM UG1, Strasbourg, Cedex, France), Enterion capsule (Pheaton Research, Nottingham, United Kingdom), and the IntelliSite capsule (Innovative Devices, Raleigh, NC, United States), have been developed for targeted drug delivery in specific areas of the GI tract[68]. However, capsule tracking is inaccurate due to lack of an anchoring mechanism and thus, drug release cannot be fully controlled. Two therapeutic capsule endoscopes have recently been proposed for bioadhesive patch release and targeted drug delivery, respectively, both capsules being controlled by an external permanent magnetic source[83,87]. A soft magnetically actuated capsule, capable of multimodal gradual or sudden drug release, has also been developed[88].

Even with a new CE designed for targeted drug delivery, several other problems should be taken into consideration. Thus, in some diseases of GI tract such as CD, drug delivery is required on a daily basis, for several days or even weeks. To overcome this problem, a pre-programmed non-viewing capsule for targeted drug delivery has been proposed[89].

Luminal insufflation

CE visualization, especially of the colonic mucosa, is limited as the capsule is unable to provide insufflation in order to distend the intestine and expose all mucosal surfaces for examination. This shortcoming is a potential cause for CE's high false-negative diagnostic rate in the colon. Experiments and insufflation capsule prototypes show the feasibility of generating large volumes of gas from a small volume of liquid hydrogen peroxide, weak acids and bases in a capsule to provide wireless insufflation for enhancing visualization[90,91].

Recently, a method of controlled colonic insufflation (CO_2) *via* an untethered capsule *in vivo* has been reported[67].

Shorter reading time

Future CE should allow shorter reading time for interpretation of images acquired by capsule, and this may be achieved by development of more efficient software[92]. A computer-aided lesion detection will significantly reduce reading time.

Home procedure

In the near future, CE (small bowel capsule endoscopy and especially colon capsule endoscopy) will become a home procedure that could be done on weekends, thus avoiding work absence[93,94].

Accurate location of detected lesion

A tagging module consisting of a micro tag, compressed spring and thermal ignitor can be integrated within future CEs; when activated by an external signal, the micro tag is impaled into the mucosa to mark the precise location of a lesion for the following endoscopic therapy[95]. Location of lesion and estimation of its size is possible by using Rapid 6 system of software developed by Given Imaging[96].

Automated capsule localization

Automated capsule localization with a software using color image analysis to discriminate between different segments of GI tract (esophagus, stomach, small bowel, colon) identified CE passage across the pylorus in 93% of cases[97-99]. The next step will be development of the software program to increase the frame rate while CE is traversing the duodenum, in order to improve identification of the ampulla of Vater and detect more lesions in the periampullary region[100,101] which is poorly visualized by CE, CT- and MR-enterography[102,103].

Entire GI tract visualization

An ideal CE would be able to visualize the entire GI tract, from mouth to anus, during a single procedure. Currently available capsules cannot be used for this purpose because of the significant physiological differences of the various segments of the GI tract, and therefore, only specific esophageal, small bowel, and colon capsules are available. However, the colon capsule (PillCam COLON 2, Given Imaging) developed for evaluation of the colon, can also be used to visualize almost the entire GI tract. This capsule is provided with two cameras able to record video images from both ends, with an adaptive frame acquisition rate (between 4 and 35 frames per second). Thus, it may visualize the esophagus, examine the stomach and duodenum with an external maneuvering system to control capsule locomotion, then the small intestine and, finally, the colon. Preliminary studies have already concluded that GI tract evaluation with PillCam COLON 2 is feasible, especially for small bowel, although other segments (esophagus, stomach) need technical improvements to obtain a good visualization[46]. In the near future, a pan-endoscopy with CE may be a reality[29,104,105].

CONCLUSION

Undoubtedly, CE has opened a new era in endoscopic diagnosis for gastroenterologists and has set a milestone in the evolution of endoscopic examination of the GI tract without discomfort or need for sedation, or the risks implied by conventional endoscopy. During a relatively short period of time (15 years), CE has proven its high diagnostic yield in multiple pathologies of the GI tract such as obscure GI bleeding, CD, celiac disease, as well as in small and large bowel tumors. Nevertheless, the endoscopic capsules currently available are diagnostic tools only, and still have several limitations (passive locomotion, inability to perform biopsy or deliver therapy, *etc*). Modern technology continues to make tremendous progress in CE, helping it overcome the above mentioned limitations. Although it is difficult to make predictions about the future, we believe that in the next 15 years, our dreams of an efficient diagnostic and therapeutic CE for the diverse pathologies of the entire GI tract will become a reality.

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REVIEW

Proteoglycans in liver cancer

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Abstract

Proteoglycans are a group of molecules that contain at least one glycosaminoglycan chain, such as a heparan, dermatan, chondroitin, or keratan sulfate, covalently attached to the protein core. These molecules are categorized based on their structure, localization, and function, and can be found in the extracellular matrix, on the cell surface, and in the cytoplasm. Cell-surface heparan sulfate proteoglycans, such as syndecans, are the primary type present in healthy liver tissue. However, deterioration of the liver results in overproduction of other proteoglycan types. The purpose of this article is to provide a current summary of the most relevant data implicating proteoglycans in the development and progression of human and experimental liver cancer. A review of our work and other studies in the literature indicate that deterioration of liver function is accompanied by an increase in the amount of chondroitin sulfate proteoglycans. The alteration of proteoglycan composition interferes with the physiologic function of the liver on several levels. This article details and discusses the roles of syndecan-1, glypicans, agrin, perlecan, collagen XVIII/endostatin, endocan, serglycin, decorin, biglycan, asporin, fibromodulin, lumican, and versican in liver function. Specifically, glypicans, agrin, and versican play significant roles in the development of liver cancer. Conversely, the presence of decorin could potentially provide protective effects.

Key words: Cancer; Cell regulation; Heparan sulfate; Liver; Proteoglycans

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Core tip: Proteoglycans are molecules that contain at least one glycosaminoglycan chain and are primarily found on the cell surface and in the extracellular matrix, where they serve as structural components. In addition, their glycosaminoglycan chains interact with numerous regulatory molecules, thus potentially influencing a myriad of cellular processes, including those linked with cancer development. For example, they can support or inhibit signaling of growth factors, cytokines, and hormones. This article reviews current data demonstrating the versatile role of proteoglycans in the development, maintenance, and progression of

liver cancer.

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INTRODUCTION

Proteoglycans are molecules with glycosaminoglycan (GAG) chains covalently attached to the protein core and are typically found on the cell surface or in the extracellular matrix (ECM). They were believed to be responsible for the maintenance of tissue turgor in the interstitial matrix, and were thought to act as molecular sieves in basement membranes. Although their sugar chains hindered the determination of their exact structure[1,2], gene technologies introduced in the second part of the last century provided a clearer picture. DNA sequencing of the regions encoding the protein cores led to the identification of glycanation sites. It then became possible to classify proteoglycans, initially based on sugar chains, and then subsequently by protein structure and function. It became clear that both the sugar chains and the protein cores of proteoglycans possess distinct and well-determined functions that are independent of each other.

The GAG chains of proteoglycans are comprised of repeating disaccharide units, each containing a uronic acid and an acetylated or sulfated hexosamine (D-glucosamine, N-acetyl-D-glucosamine, or N-acetyl-D-galactosamine). The length of these chains is variable, though the number of chains that attach to the protein core is determined by the number of sugar attachment sites, marked by Ser-Gly dipeptide motifs. Whereas the backbone of heparan sulfate (HS) is comprised of glucuronic acid and N-acetyl-D-glucosamine, chondroitin sulfate (CS) contains glucuronic acid and N-acetyl-galactosamine, and dermatan sulfate (DS) contains iduronic acid and N-acetyl-galactosamine. Instead of uronic acid, keratan sulfate (KS) contains sulfated galactose with N-acetyl-glucosamine residues. Partial sulfation of D-glucosamine residues in HS chains create a domain structure of alternating N-acetylated and N-sulfated regions. The latter can potentially interact with growth factors, cytokines, growth factor receptors, lipoproteins, and viruses, among others.

With the classification of proteoglycans (Table 1), it became evident that CS and DS proteoglycans primarily reside within the connective tissue ECM (bone, joints, tendons), whereas a considerable number of HS proteoglycans reside on the cell surface. Currently, more than 40 proteoglycans have been discovered, though only a few have been studied in the liver. This review describes what is currently known about the proteoglycans, with particular focus on the role of these molecules in the healthy and cancerous liver.

GAGS IN THE LIVER

As a parenchymal organ, the liver contains a limited amount of stromal components. As a result, HS would likely be the major GAG present on the surface of hepatocytes in normal conditions. Indeed, immunohistochemical analyses demonstrate positivity with anti-HS antibodies along the sinusoids and on the surface of hepatocytes[3]. Moreover, electrophoresis of GAGs isolated from normal human liver confirms that the majority of GAG chains are HS, with additional minor amounts of DS; CS is barely detectable (Figure 1).

The development of liver cancer is accompanied by dramatic changes in both the quantity and the composition of liver GAGs. The most conspicuous change is a 20-fold increase in CS, though enhancement of other GAGs has also been observed (Figure 1)[4]. This progressive increase in CS is accompanied by a relative decrease in HS[5]. Surprisingly, there is also a substantial increase in the amount of GAG components that appear in the seemingly normal peritumoral tissue. The alteration of GAG composition is a consequence of remodeled proteoglycan profiles, though it is not known which types are responsible. Glypican-3 and agrin are the two major HS proteoglycan components in hepatocellular carcinoma (HCC), while the source of CS in liver cancer is presumably versican.

STRUCTURAL AND FUNCTIONAL HS ALERATIONS IN LIVER CANCER

It has been reported that, unlike in normal liver, HS obtained from cancerous liver is undersulfated[6]. However, in our own experiments, although we observed a modest but significant decrease in 6-O sulfation and an increase in 3-O sulfation, total HS sulfation levels did not differ between normal liver and HCC[7]. This implies that any observed functional differences are likely based on more subtle structural alterations, such as those affecting the relationship between sulfated and acetylated domains. In addition, HSs isolated from HCC are increased in size compared to those isolated from the apparently normal peritumoral tissue[8]. It is likely that there are additional, as of yet undetermined, alterations responsible for the functional changes[9].

The diversity of ligand-binding properties on cell-surface HS proteoglycans derives from the intricate and diverse structures of their sugar chains[10]. Tyrosine kinase receptor ligands can bind with cell-surface HS to form a ternary receptor complex. At the same time, free HS proteoglycans in the ECM can compete for these binding partners, thereby creating

Table 1 Classification of proteoglycans

Localization	Eponym	Gene symbol	GAG
Intracellular	Serglycin	SRGN	Hep
Membrane			
SLIPs			
	Syndecan-1	SDC1	HS/CS
	Syndecan 2-4	SDC2-4	HS
GRIPs			
	Glypican 1-6	GPC 1-6	HS/CS
Other			
	Betaglycan	TGFBR3	HS/CS
	CD44	CD44	CS
Extracellular			
SLRPs			
Class Ⅰ			
	Decorin	DCN	DS/CS
	Biglycan	BGN	CS
	Asporin	ASPN	-
Class Ⅱ			
	Fibromodulin	FMOD	KS
	Lumican	LUM	KS
	Keratocan	KERA	KS
	PRELP	PRELP	-
	Osteoadherin	OMD	KS
Class Ⅲ			
	Epiphycan	EPYC	CS/DS
	Osteoglycin	OGN	-
Pericellular			
BM zone			
	Agrin	AGRN	HS
	Collagen XVIII	COL18A1	HS
	Aggrecan	ACAN	CS/KS
Hyalectans			
	Versican	VCAN	CS
	Neurocan	NCAN	CS
	Brevican	BCAN	CS

SLIPs: Syndecan-like integral membrane proteoglycans; GRIPs: Glypican-related integral membrane heparan sulfate proteoglycans; SLRPs: Small leucine-rich proteoglycans; BM: Basement membrane.

a soluble factor concentration gradient within the pericellular space. Moreover, many growth factors can bind HS, including basic fibroblast growth factor (bFGF), hepatocyte, platelet-derived, and vascular endothelial growth factors, and transforming growth factor (TGF)-β[11-13]. Interactions between HS and cytokines, such as regulated on activation normal T cell expressed and secreted (RANTES; also known as CCL5) and stromal cell-derived factor 1 (also known as CXCL12), have been implicated in hepatoma cell line invasion[14,15].

One of the best-known etiologic factors of HCC is hepatitis C virus (HCV) infection. Importantly, it has been shown that of HSs prepared from various bovine tissues, only those from the liver have a high affinity for E1 and E2, the envelope glycoproteins of HCV[16]. These data suggest that surface HS proteoglycans on liver cells are responsible for the liver-specific tissue tropism of HCV infection.

HSs extracted from healthy liver tissue can inhibit topoisomerase Ⅰ and Ⅱ activity[9,17] and compete with DNA for binding to several transcription factors (AP1, Ets1, TFIID, and Sp1), whereas HSs obtained from HCC do not[8]. This is important, as labeled HS proteoglycans have been shown to enter the nucleus of hepatoma cells[8]. Although the mechanism for this nuclear translocation is not known, complex formation with growth factors such as bFGF may facilitate this. Furthermore, recent data show that free or protein core-bound HSs are capable of inhibiting histone deacetylase activity, such as with syndecans shed from tumors that are taken up by stromal cells[18,19].

TRANSMEMBRANE PROTEOGLYCANS

Syndecan-1

Syndecans are a family of molecules comprised of three domains, including highly conserved intracellular and transmembrane domains and an extracellular domain that is unique to each member. The extracellular domain carries three HS chains, but a single CS chain can also be present on the core protein[20]. Syndecan-1, the most widely studied of the syndecans, is found in low amounts on the basolateral surface of hepatocytes. It is likely to be the major cell-surface HS proteoglycan in healthy liver tissue. HS chains of syndecan-1 confer all the aforementioned functions that characterize HSPGs in general. In addition it has been shown to play a critical role in lipoprotein clearance, acting as a low-density lipoprotein receptor on hepatocytes[21].

Although syndecan-1 expression is upregulated in human liver cirrhosis, our immunohistochemical analyses failed to reveal tumor-specific changes in liver cancer (unpublished data) (Figure 2). Thus, elevated syndecan-1 expression appears to be more closely associated with liver cirrhosis, rather than malignant transformation. Consistent with this idea, mRNA expression of syndecan-1 was decreased in 12 HCC samples[22], and the protein expression was low in 57 patients with invasive HCC[23].

It is important to note that the modest changes observed in syndecan-1 on the surface of hepatoma cells are likely underestimating the true effect, as a considerable proportion of syndecan-1 is shed from the cell surface. This shedding can be detected as an increased serum concentration, which also correlates with advanced Barcelona Clinic Liver Cancer stage, and therefore associated with the progression of HCC[24] and a greater risk of death[25]. As a result, expression of syndecan-1 on the surface of hepatoma cells does not reflect the role this molecule plays in the progression of liver cancer. The importance of shedding is highlighted by the work of Ramani *et al*[26], who demonstrated the importance of heparanase in the modification of HS chains and shedding of syndecan-1, which triggered the production of matrix metalloproteases. Furthermore, our unpublished data indicate that inhibition of syndecan-1 shedding in HepG2 cells induces cell differentiation *via* downregulation of the transcription factor Ets-1 and the major sheddase MMP7. Additional *in vitro* experiments

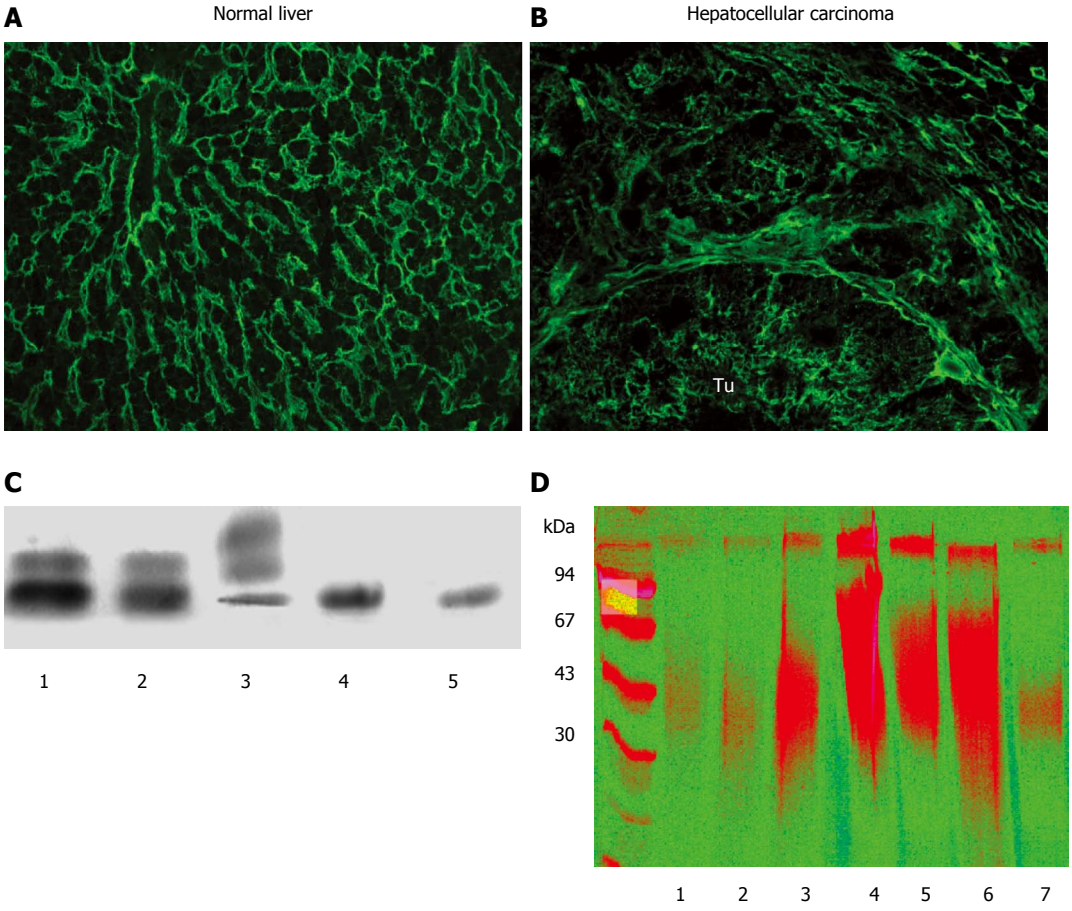

Figure 1 Expression of proteoglycans in normal and diseased liver. Detection of heparan sulfate (HS4C3 antibody) in normal liver (A) and in liver cancer (B). In normal liver the reaction is localized mainly in the sinusoids, and delicate staining is visible on the surface of hepatocytes. In contrast, in hepatocellular carcinoma (HCC) amplified amounts of heparan sulfate (HS) are visible around the individual tumor cells, most likely representing the sugar component of cell surface HSPGs. Connective tissue surrounding the tumorous nests is also loaded with HS; C: Typical picture of glycosaminoglycan (GAG) on cellulose acetate electrophoresis. GAG isolated from normal liver (1), from peritumoral liver (2), from liver cancer (3), from normal liver after chondroitinase ABC digestion (4, 5). Bands from the bottom to the top: heparan sulfate, dermatan sulfate, chondroitin sulfate. Note that the majority of GAGs are HS in the normal and peritumoral liver, whereas CS dominates in HCC; D: Separation of proteoglycans of liver origin on PAGE. Liver surrounding colon cancer metastasis (1), healthy liver (2), liver from α1-antitrypsin deficiency (3), fibrolamellar carcinoma (4), peritumoral liver of FLC (5), HCC (6), and peritumoral liver of HCC (7). Artificial colorization emphasizes the difference between normal and diseased specimens.

indicate that specific domains of syndecan-1 exert particular effects on hepatoma cells, as HepG2 and Hep3B cells expressing a truncated form of syndecan-1 (in which the extracellular domain contains only the four membrane-proximal amino acids) are induced to differentiate (unpublished data).

Glypicans

Glypicans (GPCs) are a family of six medium-sized HS proteoglycans that are tethered to the cell surface with a GPI anchor. These proteins share conserved structural features, including insertion of two to four HS chains close to the membrane, and are considered regulators of Wnt, Hedgehog, FGF, and bone morphogenetic protein signaling[27]. Although the roles of GPC-1 and GPC-5 have also been investigated in development and cancer[28-31], GPC-3 is by far the most thoroughly studied member of this family, and appears to be a key driver of hepatocarcinogenesis. Mutations of GPC-3 were first described in Simpson-Golabi-Behmel syndrome, an X-linked disorder characterized by pre- and postnatal overgrowth[32]. As these were loss-of-function mutations, it was hypothesized that the function of GPC-3 is to suppress tissue growth. In the following years, GPC-3 became increasingly implicated in cancer, and is now regarded as a typical oncofetal protein that is widely expressed during development, but silenced in adult tissues.

GPC-3 expression is elevated in several cancer types, such as embryonic tumors[33,34], malignant melanoma[35], and, most notably, HCC[36,37]. On the other hand, it is downregulated in malignant mesothelioma and ovarian cancer[38,39], and silenced *via* promoter hypermethylation in the majority of breast cancers[40,41]. The effects of GPC-3, such as the growth-inhibitory potential, have been associated with the negative regulation of Hedgehog signaling[42]. Conversely, GPC-3 may also stimulate cell proliferation by enhancing activity of the Wnt pathway. Stabilization of Wnt binding to its receptor, Frizzled, appears to be pivotal in promoting hepatocarcinogenesis[43]. It was also recently shown that GPC-3 promotes the

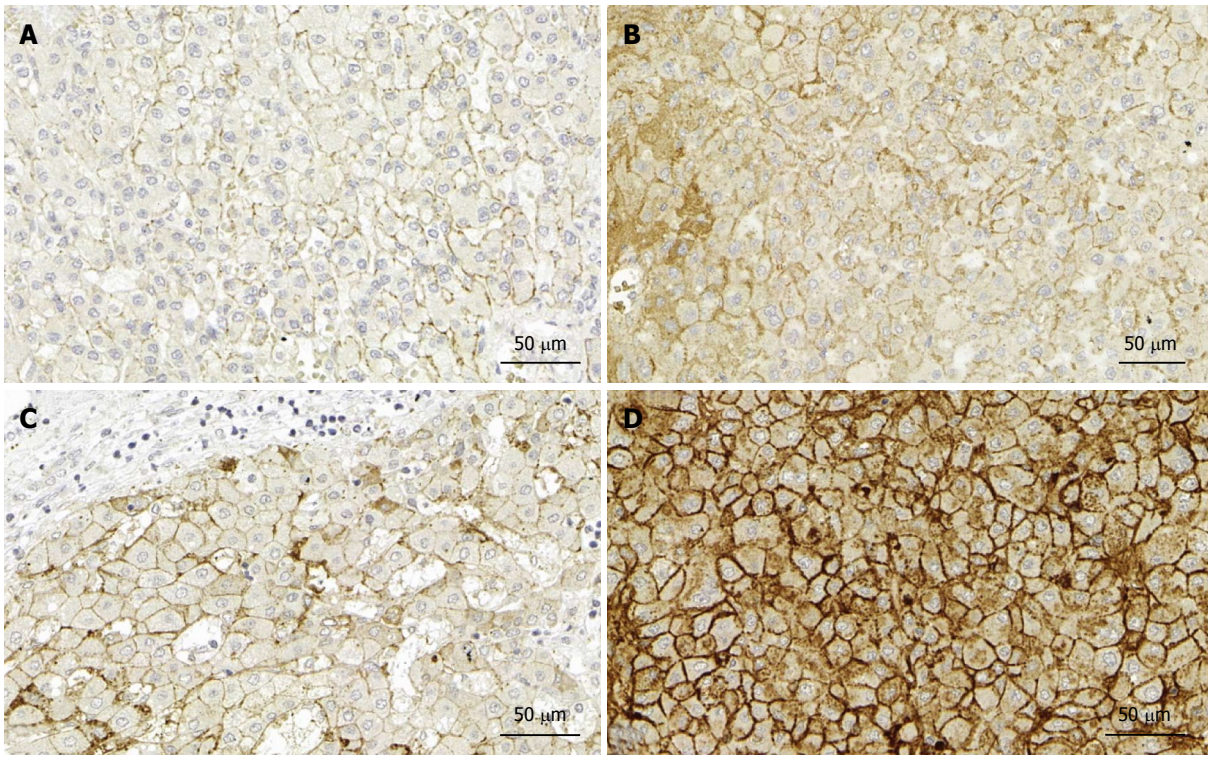

Figure 2 Syndecan expression in the liver. A: Expression of syndecan-1 in normal liver; B: Expression of syndecan-1 in liver cancer without cirrhosis; C: Expression of syndecan-1 in liver cirrhosis; D: Expression of syndecan-1 in liver cancer with cirrhosis. Note that only modest elevation of syndecan expression can be observed in cancer without cirrhosis, in contrast with the cancer specimen with cirrhosis. Immunopositivity in the cytoplasm of the latter indicates the impairment of transport to the cell surface. Immunohistochemistry on formalin fixed paraffin embedded specimens. (Primary antibody Dako MI 15).

epithelial-to-mesenchymal transition and invasive behavior of HCC cells through the stimulation of extracellular signal-regulated kinase (ERK)[44].

GPC-3 is not expressed in the normal liver[45], and overexpression in this organ is restricted to malignant hepatocellular lesions[36,46,47]. Because of this, positivity for GPC-3[48,49], along with arginase-1, CD34, glutamine synthetase, HepPar1, and heat shock protein-70[50-53], are utilized in immunohistochemical analyses for the diagnosis of HCC. Indeed, the use of various combinations of these markers when analyzing problematic samples, such as core biopsies and fine-needle aspiration specimens, can aid in resolving diagnostic dilemmas, such as distinguishing small well-differentiated HCC from dysplastic nodules[51,52,54]. Furthermore, GPC-3 can be used as a serum marker, as it is cleaved from the cell surface by the lipase notum[55]. However, GPC-3 should not be relied upon as a single serum marker, as it has only moderate accuracy in this application[37,56]. Nevertheless, GPC-3 is an attractive therapeutic target for liver cancer because of its high expression in the majority of HCCs and its known oncogenic role *via* Wnt and ERK signaling. As such, several preclinical and clinical studies have been initiated to test the efficacy of GPC-3-specific antibodies in advanced HCC patients. Although a promising candidate, the humanized mouse antibody GC33 recently failed in a phase Ⅱ trial (Yen *et al*, *J Clin Oncol* 2014; 32 Suppl 5; abstr 4102), others are still in development[57]. Additionally, immunotherapy with T cells bearing chimeric-antigen receptors specific for GPC-3 is currently being tested in a phase Ⅰ clinical trial (ClinicalTrials.gov ID: NCT02395250).

PERICELLULAR PROTEOGLYCANS

Agrin

Agrin is a large, multidomain proteoglycan with a about 220 kDa protein core that is both N-glycosylated and heavily O-glycanated with HS (and, to a lesser extent, CS) chains, thus rendering its apparent molecular mass > 400 kDa. Alternative splicing of the C-terminal exons imparts acetylcholine receptor clustering functionality, which was the first known role of agrin[58]. In addition, the use of alternative promoter sequences lead to tissue-specific expression of secreted and membrane-bound isoforms. Agrin cross-links the cytoskeleton of cells with the ECM, where it interacts with laminins and cell-surface receptors, including integrins, α-dystroglycan, and the lipoprotein-related receptor 4/muscle-specific kinase complex[58,59]. Like other HS proteoglycans, agrin binds to and regulates growth factors such as bone morphogenetic proteins and TGF-β1[60].

Since its discovery as a key motoneuron-derived synaptic organizer at the neuromuscular junction[61], agrin has been described in numerous other anatomic locations and physiologic roles. For example, in addition

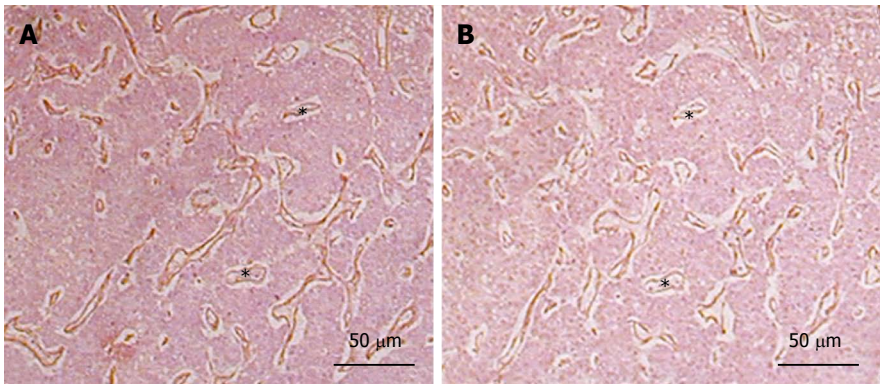

Figure 3 Colocalization of perlecan and basic fibroblast growth factor in the capillaries of hepatocellular carcinoma. A: Perlecan; B: Basic fibroblast growth factor (bFGF). Serial sections were prepared from paraffin embedded hepatocellular carcinoma specimens. Note the identical localization of perlecan and bFGF in the basement membrane of blood vessels marked by asterisks. (Primary antibodies are: mouse anti-perlecan AB: clone 7B5, Zymed Laboratories; goat anti-bFGF antibody, R&D systems).

to also organizing central nervous system synapses[62], agrin was found to orchestrate the formation of the so-called "immunologic synapse" between T cells and their antigen-presenting partners[63]. Furthermore, agrin has recently emerged as a key component of the hematopoietic stem cell niche and the matrix of peripheral lymphoid organs[64,65], and was identified as a cell-autonomous survival factor for monocytes[66]. The secreted isoform is present in specialized basal laminae, such as capillary and alveolar walls of the lung, the renal glomerular basement membrane, and the blood-brain barrier[67-70], and may be a major HS proteoglycan constituent of larger blood vessels[71].

As it is expressed only in the walls of portal arteries and the biliary compartment (comprising both mature bile ducts and the hepatic progenitor cell niche), agrin was not initially detected in healthy liver tissue[72-74]. In contrast, levels of agrin are dramatically elevated in chronic liver disease, such as cirrhosis, where it is observed in reactive bile ductules and arterial walls, and in the tumor microvasculature of HCC[73]. Indeed, its presence in tumor microvessels reliably differentiates HCC from non-malignant lesions (where it is absent), such as adenomas and dysplastic nodules, and cholangiocellular and metastatic adenocarcinomas. In non-hepatocellular adenocarcinomas, agrin is detected in pseudoglandular basement membranes rather than microvascular walls[75,76].

The origin of agrin in liver cancer and its roles in HCC progression remain unknown. However, a recent report found that multiple HCC cell lines highly express and secrete agrin[77]. In these cell lines, agrin promoted proliferation, migration, invasion, and epithelial-to-mesenchymal transition *via* the lipoprotein-related receptor 4/muscle-specific kinase complex and focal adhesion signaling. Similar protumorigenic effects of agrin (and perlecan) were also observed in oral squamous cell carcinoma[78]. Of note, the microvascular basement membrane where most agrin resides in HCC contacts other cell types in addition to the tumor cells, including endothelial cells and pericytes, which also contribute to the ECM. Furthermore, these cell types (*i.e.,* cultured rat hepatic myofibroblasts corresponding to pericytes and endothelial cells from the human umbilical vein) have also been shown to produce agrin[73,79]. It is important to acknowledge the potential for additional functions of agrin. For example, agrin may modulate vascular barrier properties of the endothelial basement membrane by reorganizing junctional complexes[80].

Perlecan

Perlecan is another large, multidomain HS proteoglycan that is synthesized by hepatic stellate cells and endothelial cells and is present in basement membranes of blood vessels and bile ducts in the portal area, as well as along the sinusoids[81]. It is structurally similar to agrin, and a transmembrane variant has also been described[82,83]. The primary functions assigned to perlecan have been attributed to the growth factor-binding HS chains, though the modular protein core also participates in numerous cell-matrix and matrix-matrix interactions[84].

Perlecan deposition is enhanced by sinusoidal capillarization during fibrogenesis[81]. In liver cancer, it becomes a component of the tumorous stroma, where it colocalizes with bFGF, indicating a possible role in tumor neoangiogenesis (Figure 3). Indeed, perlecan is known to play a central role in pathologic angiogenesis based on the high capacity of its HS chains to bind the two major proangiogenic factors, vascular endothelial growth factor and bFGF[85]. Intriguingly, perlecan can also inhibit angiogenesis *via* its C-terminal proteolytic fragment, termed endorepellin[86]. Endorepellin binds to the master matrix receptor $\alpha 2\beta 1$-integrin on endothelial cells, triggering disruption of the cytoskeleton and blockade of migration and survival[87]. Nevertheless, there are experimental and clinical data demonstrating the proangiogenic role of perlecan in human malignancies, where it promotes tumor progression in carcinomas of the breast, prostate, and colon, as well as in metastatic melanoma[88]. Thus,

perlecan is also likely to favor neovessel formation in HCC progression, though this has not yet been specifically examined.

Collagen XVIII/Endostatin

Collagen XVIII is a large basement membrane molecule that shares features with both collagens and proteoglycans. As a proteoglycan, collagen XVIII harbors several HS GAG attachment sites. At the same time, it is structurally similar to collagen XV[89,90], and has three variant forms that arise due to alternative transcription and splicing. Short variants of collagen XVIII are synthesized by the bile duct epithelial, endothelial, and vascular smooth muscle cells, and are primarily expressed in vascular, epithelial, muscle fiber, and peripheral nerve basement membranes. Long variants are mainly produced by hepatocytes and deposited in the perisinusoidal space. However, activated hepatic stellate cells also produce the short collagen XVIII variant during fibrogenesis, which then becomes a major component of the altered basement membrane in capillarized sinusoids[91] and of the ECM in primary and metastatic liver cancers[92]. At the same time, the mRNA levels of collagen XVIII are decreased in liver cancer, and have been associated with larger tumor size, higher microvessel density, and recurrence after tumor resection[93]. It has been proposed that a decrease in the long variant, which contains a domain homologous to the Wnt-binding Frizzled receptor, favors tumor progression in HCC[92,94] by promoting activation of the Wnt/β-catenin pathway[95].

The C-terminal domain of collagen XVIII can be cleaved by several proteases, including MMP-3, -7, -9, -13, -20, elastase, and cathepsin L, producing a proteolytic fragment called endostatin, which has a strong antiangiogenic effect[90]. Endostatin influences several signaling pathways, including vascular endothelial growth factor, Wnt/β-catenin, and α5β1-integrin, which are known to play crucial roles in hepatocarcinogenesis. Exogenous administration of endostatin has also been shown to inhibit tumor growth in various tissues, as well as HCC[96]. These features led to the use of endostatin as a therapeutic agent for cancer in phase Ⅰ and Ⅱ clinical trials, though the results were less promising than were found in preclinical experiments. Nevertheless, as stable disease and regression were observed in several cases, the use of a modified form of endostatin has continued in certain countries for the treatment of lung and gastric cancers[97]. Additionally, endostatin can be detected in serum, and therefore could be used for predicting prognosis[93,98]. For example, preoperative serum endostatin is inversely correlated with microvessel density in HCC patients and is related to a better prognosis after resection[93].

Endocan

Endocan, a proteoglycan whose core protein is glycanated by a single DS chain, is typically produced by endothelial cells[99]. Endocan is not normally expressed in highly vascularized organs such as the liver, but is rather synthesized upon inflammation and malignant transformation, indicating that it may be a characteristic feature of activated endothelial cells[100]. In liver cancer, endocan expression is confined to the microvessels of the tumor tissue, but is not present in capillaries in the peritumoral liver[101]. The DS chain of endocan has a high affinity for hepatocyte growth factor, an interaction that is critical for the activation of the Met receptor, which stimulates proliferation of hepatoma cells[100].

When analyzing the factors associated with clinicopathologic characteristics of HCC tumors, it was found that endocan-positive, but not CD34-positive, microvessel density was strongly correlated with microscopic venous invasion, a reliable indicator of postoperative recurrence[101]. Increased production of endocan can be detected in serum, which also can be used as a prognostic indicator, as it is associated with poor hepatic function, a greater number of tumors, and vascular invasion[102].

INTRACELLULAR PROTEOGLYCANS

Serglycin

One of the first to be sequenced, serglycin is the only proteoglycan with intracellular localization. Another unique feature of serglycin is that it has heparin as a sugar side chain, which allows it to interact with a variety of inflammatory mediators, such as proteases, cytokines, and growth factors[103,104]. Serglycin is mainly produced by hematopoietic cells, and it was only recently discovered that it is also expressed by tumor cells, where it promotes their aggressive behavior[104]. Consistent with this, overexpression of serglycin in HCC is predictive of a poor prognosis, with increasing levels corresponding to advanced Barcelona Clinic Liver Cancer staging, vascular invasion, and early recurrence[105]. In addition, the increased level of serglycin correlates with elevated vimentin and reduced E-cadherin levels, and is therefore thought to induce epithelial-to-mesenchymal transition in HCC[105].

SMALL LEUCINE-RICH PROTEOGLYCANS

Small leucine-rich proteoglycans (SLRPs) represent the largest family of proteoglycans, with 18 distinct gene products and several splice variants. Members of this proteoglycan family are characterized by a relatively small core protein comprised of a central region of leucine-rich repeats[103,106]. Although most SLRP members carry CS/DS or KS chains, there are a few

that lack GAG chains, and are thus only classified as proteoglycans based on their structural and functional homology with other SLRP members[91,103,107].

Decorin

Decorin is the prototypical member of the SLRP family, and is glycanated with either a single CS or DS chain[106,108]. Primarily expressed by fibroblasts and myofibroblasts among various tissue types, decorin is most abundant in the skin, connective tissues, muscles, and the kidney[109]. Indeed, it was named based on its "decoration" of collagen fibers through binding to collagen I where it regulates fibril formation and stabilization[110,111]. However, it is also known to be involved in many biologic and physiologic processes, including cell proliferation and differentiation, *via* interaction with various growth factors, matrix constituents, and signal transduction pathway-coupled receptors, such as those known to be dysregulated in HCC[112]. Therefore, soluble forms of decorin may act as pan-receptor tyrosine kinase inhibitors, targeting receptors for epidermal growth factor, HGF, insulin-like growth factor, vascular endothelial growth factor, and platelet-derived growth factor[108,113-121]. Interactions with such receptors can also lead to decorin-induced caveosomal internalization and receptor degradation[122].

In the healthy liver, decorin levels are generally low, with the exception of the central veins and the portal tracts where expression is higher. Some expression has also been detected in the sinusoidal walls. However, decorin accumulates with increased connective tissue production in chronic liver injury and is deposited along the sinusoidal walls as capillarization occurs, though its appearance often precedes the accumulation of fibrillar collagens. Furthermore, decorin colocalizes with high amounts of TGF-β1, a key stimulator of fibrillogenesis, in fibrotic areas in cases of chronic hepatitis, fibrosis, and cirrhosis[123]. Indeed, decorin has been shown to directly bind to and regulate TGF-β1[124,125], as well as indirectly *via* association with the low-density lipoprotein receptor-related protein receptor[126]. Through similar interactions, decorin can inhibit TGF-β1-dependent proliferation of fibroblasts, production of matrix in experimental renal fibrogenesis[127], and collagen synthesis in hepatic stellate cells[128]. Moreover, decorin-deficient mice exhibit increased susceptibility to thioacetamide-induced fibrogenesis, as well as enhanced connective tissue deposition and significantly higher TGF-β1 bioactivity compared to their wild-type counterparts[129]. The lack of decorin also impeded the resolution of severe fibrosis, which may have been due to impaired matrix metalloprotease action. In addition, *in vitro* experiments demonstrated that knockdown of decorin in the LX2 hepatic stellate cell line increased the expression of smooth muscle actin and collagen type I. These observations support the notion that decorin could be utilized as an anti-TGF-β1 agent in the treatment of chronic liver diseases[130]. However, it is important to note that decorin can be present in both soluble and collagen-bound pools, which could potentially modulate its antifibrogenic activity.

The observation that decorin can affect multiple signaling pathways dysregulated in HCC[112], along with its ability to downregulate β-catenin and Myc expression[131] with concurrent induction of $p21^{WAF1/CIP1}$[132], indicates that decorin may serve as a tumor suppressor in liver cancer. In support of this role, levels of decorin mRNA are reportedly downregulated in HCC[133,134], and decorin was found to inhibit proliferation of HuH7[135] and HepG2 hepatoma cells *via* induction of $p21^{WAF1/CIP1}$ and $p57^{KIP2}$[136,137], while enhancing apoptosis[135-137]. Furthermore, virus-mediated transfection of decorin causes death of xenografted HCC cells[138].

Interestingly, well-defined deposits of decorin surround tumor foci in experimental models of hepatocarcinogenesis[139]. At the same time, decorin production is decreased in the majority of human hepatoma stroma cells within the tumor tissue, while the expression is increased in the surrounding connective tissue (unpublished data). Furthermore, an inverse correlation exists between decorin expression and aggressive HCC behavior, as ablation of the decorin gene results in enhanced tumor formation in primary hepatocarcinogenesis models, likely due to enhanced receptor tyrosine kinase signaling and failed $p21^{WAF1/CIP1}$ induction[119,139]. Indeed, increased phosphorylation of epithelial and platelet-derived growth factor receptors, macrophage stimulating protein receptor, and insulin-like growth factor receptor was observed with decorin deletion[139].

Biglycan

Biglycan is a member of the SLRP family that is produced by activated hepatic stellate cells during fibrogenesis[140,141]. Biglycan is deposited along with decorin in fibrotic areas[142], and similarly interacts with members of the TGF-β/bone morphogenetic protein family[107]. However, mechanistic details of its contribution to liver diseases have not yet been elucidated[91].

Asporin

Asporin is a proteoglycan that is very similar in structure to decorin and biglycan, though it does not contain any GAG chains[143,144]. Its name is derived from its aspartic acid-rich N-terminal region and its overall homology and similar tissue distribution to decorin. Although it is not expressed in normal liver tissue[143,144], levels of asporin are upregulated in cancer-associated fibroblasts in gastric carcinoma[145]. Moreover, asporin can interfere with TGF-β/Smad signaling[146], and therefore may potentially play a role in liver malignancies.

Fibromodulin and lumican

Fibromodulin and lumican are SLRPs that contain KS GAG chains. Although not detectable in normal liver, expression of these proteoglycans is induced during liver fibrogenesis[147]. Fibromodulin was shown to promote the profibrogenic potential of hepatic stellate cells[148], and lumican was found to be a prerequisite for hepatic fibrosis[149]. However, the role of these specific SLRPs in hepatocarcinogenesis has not yet been determined.

HYALECTANS

Versican

Versican is a member of the hyaluronic acid-binding proteoglycan group that contains CS and DS GAG side chains, and is a ubiquitous component of the interstitial stroma ECM. Its four splice variants (V0, V1, V2, and V3) all have N-terminal and C-terminal globular domains responsible for various molecular interactions, and may or may not contain two GAG binding domains (GAGα and GAGβ). In adult tissues, the V2 variant is the most frequent, which contains the CS GAGβ binding domain. The number of CS/DS chains varies among the variants, whereas the number, length, and molecular structure of the GAG chains are influenced by the N-terminal and C-terminal globular domains[150]. Synthesis of versican is primarily stimulated by TGF-β1[151-153], but also by platelet-derived growth factor and interleukin 1α, whereas its production is inhibited by interleukin 1β[154,155].

In the ECM, versican interacts with various partners, such as tenascin-R, fibulin-1[156-158], fibrillin-1[157], fibronectin[159], P- and L-selectin[160,161], and various chemokines[150] *via* the globular domains or GAG chains. In addition, versican has been shown to bind to the epidermal growth factor receptor[150], and to the cell-surface proteins CD44[160] and integrin β1[162]. Recently, versican has been shown to act on macrophages through toll-like receptors (TLR2 and TLR6), and promote inflammatory cytokine production and tumor cell metastasis[163]. The ability of versican to interact with a variety of regulatory components and cell-surface molecules indicates that versican could potentially influence cell adhesion, proliferation, apoptosis, migration, and invasion[164-167].

Elevated expression of versican (V0 and V1 variants) has been found in numerous types of malignancies, including breast and prostate cancers, gliomas, osteosarcomas, fibrosarcomas, and melanomas, where it is associated with cancer relapse and poor patient outcome[168-174]. Recent studies show that expression of versican is regulated by a number of microRNAs[175,176]. Interestingly, the 3'UTR of versican can bind to and antagonize some of these microRNAs, thereby enhancing its own expression[177]. Indeed, transgenic mice expressing the 3'UTR region of versican develop HCC[178], demonstrating a possible role for versican expression in hepatocarcinogenesis. However, versican was not detected in tumor cells in immunohistochemical analyses from a large cohort of human HCC specimens[179]. Thus, further study is needed to confirm the role of versican in liver cancer.

CONCLUSION

Proteoglycans can be found on the cell surface and in the extracellular matrix. Their GAG chains interact with numerous regulatory molecules and signaling pathways, including growth factors, cytokines, and hormones, thus affording the potential to influence a myriad of cellular processes, including those linked with cancer development. There is clear evidence that proteoglycan composition changes with liver cancer development, and thus proteoglycans provide targets for potential therapeutic agents and diagnostic biomarkers.

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REVIEW

Chemoprevention of obesity-related liver carcinogenesis by using pharmaceutical and nutraceutical agents

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Abstract

Obesity and its related metabolic disorders are serious health problems worldwide, and lead to various health-related complications, including cancer. Among human cancers, hepatocellular carcinoma (HCC) is one of the most common malignancies affected by obesity. Therefore, obesity and its related disorders might be a key target for the prevention of HCC. Recently, new research indicates that the molecular abnormalities associated with obesity, including insulin resistance/hyperinsulinemia, chronic inflammation, adipokine imbalance, and oxidative stress, are possible molecular mechanisms underlying the pathogenesis of obesity-related hepatocarcinogenesis. Green tea catechins and branched-chain amino acids, both of which are classified as nutraceutical agents, have been reported to prevent obesity-related HCC development by improving metabolic abnormalities. The administration of acyclic retinoid, a pharmaceutical agent, reduced the incidence of HCC in obese and diabetic mice, and was also associated with improvements in insulin resistance and chronic inflammation. In this article, we review the detailed molecular mechanisms that link obesity to the development of HCC in obese individuals. We also summarize recent evidence from experimental and clinical studies using either nutraceutical or pharmaceutical agents, and suggest that nutraceutical and pharmaceutical approaches targeting metabolic abnormalities might be a promising strategy to prevent the development of obesity-related HCC.

Key words: Hepatocellular carcinoma; Obesity; Green tea catechins; Branched-chain amino acids; Acyclic retinoid

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Core tip: Obesity and its related metabolic disorders increase the risk of hepatocellular carcinoma (HCC). In particular, the molecular abnormalities represented by insulin resistance/hyperinsulinemia, chronic inflammation, adipokine imbalance, and oxidative stress play a central role in the development of obesity-related HCC. Administration of green tea catechins, branched-chain amino acids, and acyclic retinoid has improved these metabolic abnormalities, and resulted

in the inhibition of HCC development in obese and diabetic mice models. In this review, we highlight the possibility that nutraceutical and pharmaceutical approaches targeting metabolic abnormalities are a promising strategy to prevent the development of obesity-related HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third leading cause of cancer-related deaths in the world. It accounts for more than 90% of primary liver cancers, and its incidence is increasing[1,2]. It is well known that HCC primarily develops from chronic liver inflammation and subsequent cirrhosis induced by persistent infection with the hepatitis B or hepatitis C viruses[3]. However, more recently, the incidence of HCC attributed to nonalcoholic fatty liver disease (NAFLD), which is related to obesity and systemic insulin resistance[4], has been rapidly increasing, especially in developed countries[5].

Obesity is now recognized as one of the most serious health problems worldwide, and its prevalence has dramatically increased in the last few decades[6]. It often causes a number of medical disorders, including type-2 diabetes mellitus, hypertension, and hyperlipidemia, which are collectively known as "metabolic syndrome". In addition, recent publications indicate that obesity and its related metabolic abnormalities, especially diabetes mellitus, are important risk factors for the development of many types of human malignancies, including HCC[7-16]. Moreover, obesity-associated neoplasms are likely to be more aggressive, and have an increased risk of recurrence, thereby resulting in higher mortality[17,18]. Indeed, in a prospective study conducted in a large cohort of American adults, Calle *et al*[19] reported that men with a body mass index (BMI) greater than 35 kg/m^2 had significantly higher mortality rates due to HCC when compared to men with a normal BMI.

Accumulating evidence from epidemiological and experimental studies indicates that several pathophysiological mechanisms link obesity to liver carcinogenesis, including insulin resistance and adipocytokine imbalance, alterations in the insulin-like growth factor-1 (IGF-1)/IGF-1 receptor (IGF-1R) axis, a state of chronic inflammation, and the induction of oxidative stress[9,10,15,20,21]. Meanwhile, several experimental studies have revealed that the improvement of chronic inflammation through the inhibition of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, plays an important role in the suppression of obesity-related colorectal cancer and HCC[22-25]. These facts suggest that pathophysiological disorders due to obesity and its related metabolic alterations could be critical targets for the chemoprevention of obesity-related liver carcinogenesis.

In this review, we summarize the multiple pathogenic mechanisms by which obesity and its related metabolic disorders induce the development of HCC, with a special focus on the emergence of insulin resistance and subsequent inflammatory cascades. We also discuss the possibility of nutraceutical or pharmaceutical approaches for targeting obesity-induced pathophysiological disorders for the prevention of obesity-related liver carcinogenesis.

LIVER STEATOSIS AND HCC

NAFLD has been referred to as the hepatic manifestation of obesity and metabolic syndrome and has become one of the most common liver diseases in developed countries[26]. NAFLD is mostly limited to liver steatosis, but approximately 20% of all cases present as nonalcoholic steatohepatitis (NASH) featuring hepatocyte injury, chronic liver inflammation, and various degrees of fibrosis with an elevated risk of developing liver cirrhosis and HCC[27-30]. Obesity is the main risk factor for NAFLD and up to 90% of obese people have some degree of liver steatosis[31]; however, NAFLD can be observed even in non-obese individuals with insulin resistance and hyperinsulinemia[4,32]. In contrast, obesity with only abdominal adiposity and without insulin resistance does not appear to play a role in liver steatosis[33]. These results indicate that obesity-induced metabolic abnormalities, especially insulin resistance, might be a crucial factor in the development of NAFLD.

Most NAFLD-related HCCs are believed to develop in the background of cirrhotic liver, similar to other etiologies such as chronic hepatitis virus infection[3]. Accumulating evidence indicates that NAFLD-induced cirrhosis increases the risk of HCC development in the absence of other risk factors[34-37]. Primary liver carcinomas, including HCC, often occur in patients with NASH, especially in those with advanced fibrosis and cirrhosis[38,39]. However, evidence is also accumulating indicating that NAFLD is strongly associated with the development of non-cirrhotic HCC[40-43].

A recent study from Yasui *et al*[44] revealed that approximately half of the 87 patients with HCC and biopsy-proven NASH had no established cirrhosis. In addition, a population-based study reported that 2863 cases of HCC (16% of the total number of HCC cases) were due to histologically confirmed NAFLD without other etiologies[45]. Notably, 1031 cases (36%) of NAFLD-related HCC were found in non-cirrhotic livers,

and 18% of these cases developed in simple fatty liver without steatohepatitis[45]. Thus, liver cirrhosis is not necessarily linked to the occurrence of obesity-related HCC.

As described above, NAFLD-related HCCs are likely to occur even in individuals with either NASH or simple fatty liver, in the absence of advanced liver fibrosis[34,40-44,46]. In these cases, the presence of metabolic syndrome, especially type 2 diabetes mellitus and obesity, plays a positive role in the development of HCC[47]. Notably, the features of HCC arising in these individuals are different from those arising in patients with chronic viral hepatitis, in terms of tumor size, the degree of tumor differentiation, and the extent of liver fibrosis[43]. Thus, given these differences between NAFLD-related and hepatitis virus-induced HCC, it is anticipated that specific pathophysiological mechanisms may present in the background of NAFLD-related hepatocarcinogenesis.

POTENTIAL PATHOPHYSIOLOGICAL MECHANISMS OF HEPATOCARCINOGENESIS ASSOCIATED WITH OBESITY

Although the pathways linking obesity to hepatocarcinogenesis remain poorly defined, accumulating evidence has led to the identification of potential pathophysiological mechanisms, including insulin resistance and the subsequent inflammatory cascades. Liver-specific and systemic insulin resistance are major consequences of obesity[20,48] and lead to fat accumulation in the hepatocyte by lipolysis and hyperinsulinemia, resulting in the development of liver steatosis, including NAFLD[32,47]. In addition, insulin resistance and hyperinsulinemia increase the biological activity of IGF-1, an important endocrine and paracrine regulator of tissue growth and metabolism[49-51].

The binding of insulin and IGF-1 to their respective cell surface receptors on tumors activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is responsible for cellular processes like growth, proliferation, and survival[52,53] Indeed, alterations in the IGF-1/IGF-1R axis have been shown to contribute to the pathogenesis of HCC[52-55]. Moreover, insulin resistance also leads to an increased expression of pro-inflammatory cytokines, including TNF-α and IL-6, through the creation of an oxidative stress environment in the tissues[56]. The dysregulation of these cytokines is associated with the development of steatosis, chronic liver inflammation, and liver tumor formation[9,10,15,20,25,57]. Furthermore, DNA damage due to increased oxidative stress activates the PI3K/Akt pathway[58,59], suggesting that both of these may promote liver tumorigenesis in obese individuals. Thus, insulin resistance and its related inflammatory cascades are thought to be key factors involved in the development of obesity-associated HCC.

Excess fat accumulation in obesity results from a chronic increase in nutrient intake and a decrease in physical exercise, which leads to expansion of adipose tissue and recruitment of various immune cells, such as macrophages[60-62]. Hypertrophic adipocytes and infiltrated macrophages secrete free fatty acids and various pro-inflammatory cytokines, including TNF-α and IL-6[9,10,15,20], and contribute to the development of insulin resistance and low-grade, chronic inflammation[63,64]. Moreover, excess production of storage lipids causes an imbalance of serum adipokine levels, which is related to obesity-associated carcinogenesis[65,66].

The serum levels of adiponectin, an anti-inflammatory and tumor growth-limiting cytokine, are reduced in obese individuals[67] and are negatively correlated with obesity[68,69]. On the other hand, high-circulating levels of leptin, a major adipokine with pro-inflammatory and pro-fibrogenic effects, are observed in patients with obesity and NAFLD[70]. Notably, leptin has a growth-promoting effect through the activation of Janus kinase (JAK), a signal transducer and activator of transcription-3 (Stat3), PI3K/Akt, and extracellular signal-regulated kinase (Erk) signaling pathways[71]. In addition, leptin can induce the expression of TNF-α and IL-6[72,73], and result in tumor growth and progression as described above. Leptin also induces oxidative stress and inflammation in endothelial cells[74]. Indeed, in HCC patients, higher levels of serum leptin increase the risk of HCC recurrence after curative treatment[75]. Moreover, the positive association between leptin levels and the development of HCC has been elucidated by recent *in vitro* studies[71,76-79]. Taken together, these facts suggest that obesity-related metabolic abnormalities work simultaneously with, and complementary to, one another, and that they increase the risk of cancer, including HCC, in obese individuals (Figure 1).

OTHER POSSIBLE MECHANISMS LINKING OBESITY TO HEPATOCARCINOGENESIS: GENETIC RISK FACTORS

Recently published research has highlighted the relevance of genetic risk factors in the predisposition toward hepatocarcinogenesis in patients with NAFLD[80]. In particular, the I148M variant of patatin-like phospholipase domain-containing protein 3 (PNPLA3) is a risk factor for HCC development in obese and NAFLD patients[81,82]. Indeed, one recent cohort study involving 3473 obese individuals observed a high incidence of HCC development in the subjects with the I148M risk allele[83]. Interestingly, this risk allele is associated with HCC development independently of its effect on the progression of liver fibrosis and cirrhosis[82,84,85]. Given that NAFLD-related HCC is likely to occur in individuals without advanced liver fibrosis, it

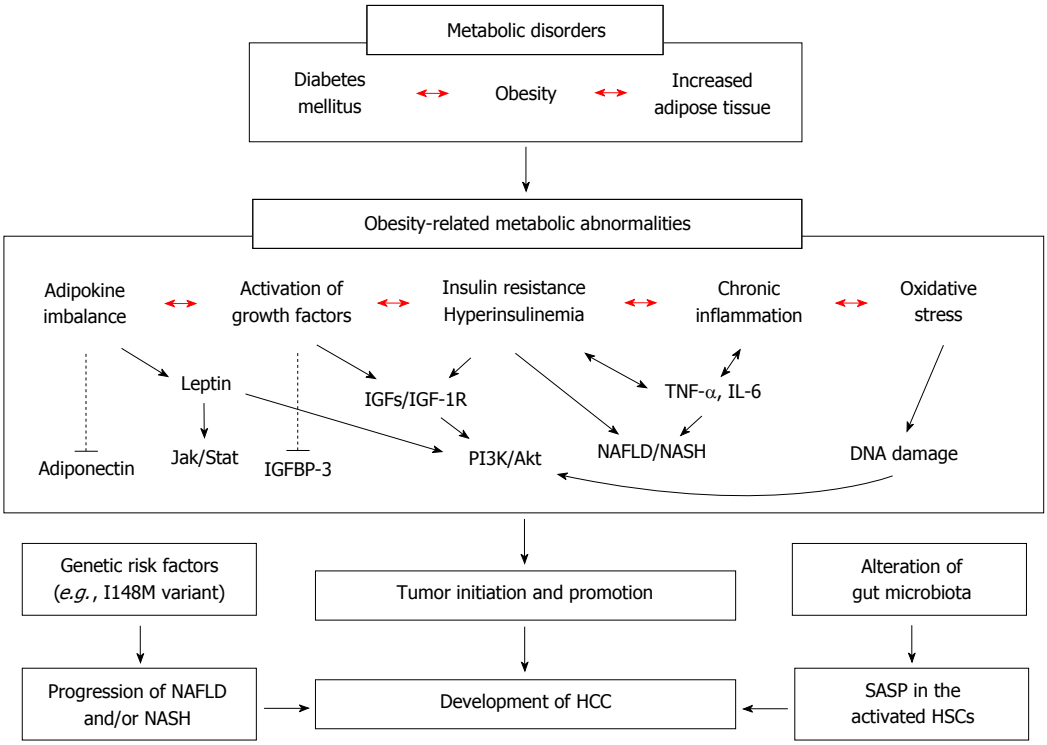

Figure 1 Proposed mechanisms linking obesity and its related metabolic abnormalities to the development of hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HSCs: Hepatic stellate cells; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IGF-1: Insulin-like growth factor-1; IGFBP-3: IGF-binding protein-3; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; Jak: Janus kinase; Stat: Signal transducer and activator of transcription; SASP: Senescence-associated secretory phenotype; PI3K: Phosphatidylinositol 3-kinase.

is believed that genetic risk factors, such as the I148M variant, also play an important role in the development of NAFLD-related HCC (Figure 1).

OTHER POSSIBLE MECHANISMS LINKING OBESITY TO HEPATOCARCINOGENESIS: MICROBIOME COMPOSITION

The relationship between the intestinal microbiome and metabolic regulation is attracting an increasing amount of attention. Indeed, several experimental studies have demonstrated that intestinal dysbiosis is associated with the development of metabolic disorders, including obesity, insulin resistance, and NAFLD[86-92]. Interestingly, obesity-induced alteration of gut microbiota promotes liver carcinogenesis through the activation of hepatic stellate cells (HSCs). Dietary and genetic obesity induced an alteration of gut microbiota, resulting in increased levels of deoxycholic acid (DCA)[93]. The enterohepatic circulation of DCA induced senescence-associated secretory phenotype (SASP) in the activated HSCs, leading to hepatocarcinogenesis via the secretion of various tumor-promoting factors in the liver[93]. Notably, the inhibition of DCA production or the reduction of gut bacteria prevented the development of HCC in obese mice[93]. Thus, these results indicate that the SASP in the activated HSCs due to obesity-induced gut microbial metabolites plays a key role in the development of obesity-related HCC (Figure 1).

BENEFICIAL EFFECTS OF WEIGHT REDUCTION IN PATIENTS WITH NAFLD

Although several agents have been evaluated in clinical trials, there are currently no well-established therapies for NAFLD[94]. However, several recent clinical studies have elucidated the beneficial effects of weight reduction in the improvement of NAFLD[95-100]. Notably, weight reduction based on dietary and lifestyle modifications improved the histological features of NAFLD in overweight subjects[101]. Interestingly, this beneficial effect was associated with an improvement in biological parameters (aspartate aminotransferase/alanine aminotransferase/γ-glutamyltransferase), metabolic ones (body mass index/fasting glucose/insulin resistance) or in the imbalance of adipocytokines[101]. Besides, recent studies examined the association between the magnitude of weight reduction and changes in histological features of liver steatosis, and reported that a weight loss of over 7% is essential to yield histological outcomes[96,97,100,102]. Moreover, Vilar-Gomez *et al*[103] reported that weight reduction of over 10% through lifestyle modification significantly reduced NASH-related histological features, including fibrosis and portal inflammation. Thus, weight reduction based on lifestyle modification can be effective in the

management of patients with NASH, and is currently recommended as a first line therapeutic intervention for this disease[94,103,104].

PREVENTION OF OBESITY-RELATED HCC USING THE NUTRACEUTICAL APPROACH: GREEN TEA CATECHINS

As stated earlier, obesity and its related metabolic abnormalities, including insulin resistance and chronic inflammation in the liver, play an important role in the development of HCC. This indicates that metabolic abnormalities induced by obesity may be a valuable target in the prevention of liver carcinogenesis in obese individuals. Indeed, genetic ablation of TNF-α and IL-6 signaling could reduce the incidence of obesity-promoted hepatocarcinogenesis through the reduction of liver steatosis and steatohepatitis[25]. In support of this idea, administration of adiponectin resulted in the reduction of leptin-induced liver tumorigenesis in nude mice[105]. Thus, targeting obesity-related metabolic abnormalities is a promising strategy for the prevention of HCC.

An improvement of metabolic abnormalities through nutraceutical or pharmaceutical intervention might be an effective strategy to inhibit obesity-related liver carcinogenesis, as has already been reported experimentally for colon carcinogenesis[106]. In order to verify this hypothesis, we experimentally investigated the chemopreventive effects of nutritional agents, including green tea catechins (GTCs) and branched-chain amino acids (BCAA) in obese and diabetic C57BL/KsJ-*db/db* (*db/db*) mice, and supplemented our findings by summarizing the relevant results of recent publications.

The *db/db* mice have a functional defect in the long-form leptin receptor, leading to hyperleptinemia and obesity due to overeating. Because of the obesity, hyperinsulinemia, and hyperleptinemia, these mice are regarded as a suitable animal model that mimics metabolic syndrome in humans[107]. In addition, the mice are susceptible to chemical carcinogens and develop *N*-diethylnitrosamine (DEN)-induced liver tumorigenesis through the activation of IGF/IGF-1R and the induction of chronic inflammation in the liver[54,55,108]. Thus, the *db/db* mice are thought to be a suitable mouse model of obesity-related hepatocarcinogenesis[54].

Recently, the beneficial effects of GTCs on the improvement of obesity have been reported[109]. A mechanistic review reported that the anti-obesity effects of GTCs results from underlying mechanisms that promote energy expenditure, fatty acid oxidation, and a reduction in nutrient absorption[110]. In addition, GTCs improved hyperglycemia, insulin resistance, and hyperleptinemia, and result in an improvement in liver steatosis and liver dysfunction in rodent diabetic models[111-113]. Treatment with GTCs decreases serum levels of insulin, TNF-α and IL-6 in insulin-resistant rats[114]. Thus, GTCs possess the ability to improve obesity and its related metabolic abnormalities.

In addition to the improvement of obesity, other studies have reported on the anti-cancer and cancer-preventative effects of GTCs[115-118]. A number of studies have demonstrated that GTCs inhibit the proliferation of, and induce apoptosis in, cancer cells by modulating the activation of several receptor tyrosine kinases (RTKs) and their downstream signaling pathways, such as Ras/Erk and PI3K/Akt[115-117,119,120]. Moreover, the down-regulation of IGF/IGF-1R and the activation of IGF-binding protein-3 (IGFBP-3), which negatively controls IGF/IGF-1R signaling, are responsible for the growth inhibition of colorectal and HCC cells[121,122]. Given that IGF/IGF-1R signaling plays an important role in the development of obesity-related HCC as stated above, GTCs are a promising candidate for the chemoprevention of this malignancy.

Indeed, our recent publication reported that (-)-epigallocatechin gallate (EGCG), a major biologically active component of green tea catechins, significantly inhibited the development of liver foci and adenoma in DEN-treated *db/db* mice[55]. Moreover, EGCG decreased the serum levels of insulin, IGF-1, IGF-2, and inhibited the phosphorylation of IGF-1R, Erk, Akt, and GSK-3β in the liver[55]. Furthermore, mRNA expression of TNF-α, IL-6, IL-1β, and IL-18 in the liver was reduced by EGCG treatment[55]. Thus, EGCG prevents obesity-related HCC development by modulating IGF/IGF-1R signaling, and by improving both insulin resistance and chronic liver inflammation. These data also indicate that GTCs, especially EGCG, may be useful for the chemoprevention of obesity-related HCC development (Figure 2).

To date there are no clinical studies that evaluate the chemopreventive effects of GTCs on obesity-related hepatocarcinogenesis in humans. However, our pilot study showed that oral supplementation of GTCs (1.5 g/d) for 1 year significantly reduced the incidence of metachronous colorectal adenomas after polypectomy[123]. In addition, a randomized, double-blinded, placebo-controlled study reported that oral administration of GTCs for 1 year prevented the progression of high-grade prostate intraepithelial neoplasia to prostate cancer[124]. Considering its chemopreventive effects in several human malignancies, an interventional approach using GTCs might also be effective in the prevention of obesity-related hepatocarcinogenesis. However, further clinical studies are needed in this field to verify our hypothesis.

PREVENTION OF OBESITY-RELATED HCC USING THE NUTRACEUTICAL APPROACH: BRANCHED-CHAIN AMINO ACIDS

As the liver plays an important role in the regulation of metabolism, patients with chronic liver disease are often

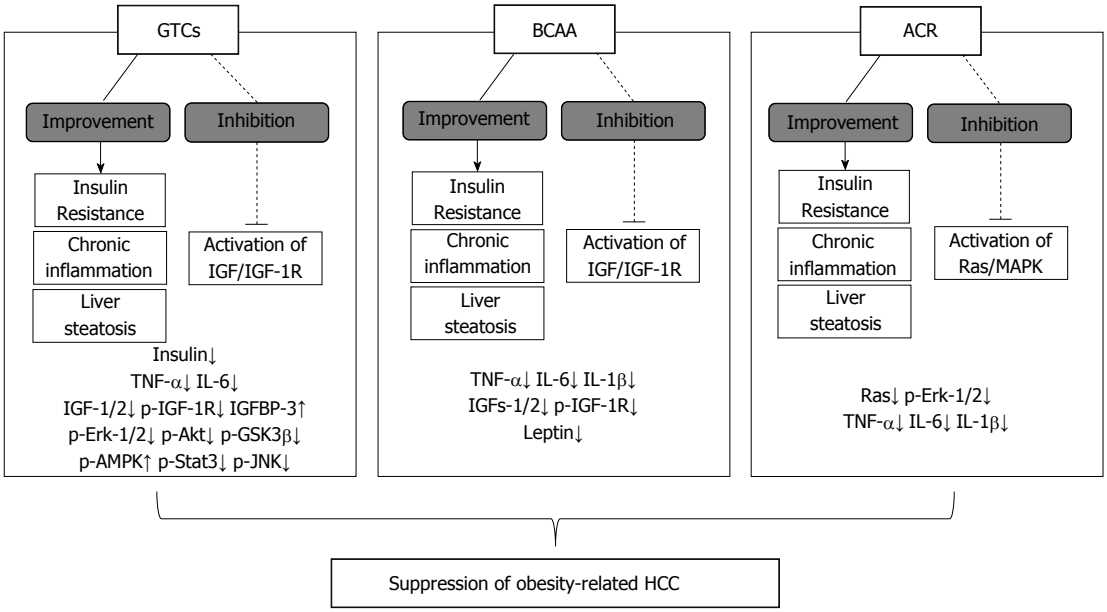

Figure 2 Mechanisms of action of green tea catechins, branched-chain amino acids, and acyclic retinoid in the inhibition of obesity-related liver carcinogenesis. HCC: Hepatocellular carcinoma; GTCs: Green tea catechins; BCAA: Branched-chain amino acid; ACR: Acyclic retinoid; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IL-1β: Interleukin-1β; IGF-1: Insulin-like growth factor-1; IGF-1R: Insulin-like growth factor-1 receptor; IGFBP-3: IGF-binding protein-3; Stat3: Signal transducer and activator of transcription-3; Erk-1/2: Extracellular signal-regulated kinase-1/2; GSK-3β: Glycogen synthase kinase-3β; AMPK: AMP-activated kinase.

malnourished and have metabolic abnormalities, such as hypoalbuminemia and insulin resistance[125-128]. The decreased serum levels of BCAA (valine, leucine, and isoleucine) and hypoalbuminemia worsen the outcomes of cirrhotic patients; however, supplementation with BCAA improves protein-energy malnutrition and hypoalbuminemia, resulting in an improvement in the quality of life and in the prognosis of cirrhotic patients[125,127,129,130]. In addition, the beneficial effects of BCAA on the regulation of glucose metabolism have been demonstrated in recent clinical and experimental studies[131-134]. Thus, BCAA possesses the ability to improve not only malnutrition induced by chronic liver diseases, but also glucose intolerance, such as insulin resistance.

Notably, a long-term survival study reported that the continuous administration of BCAA was associated with a reduced incidence of HCC in obese cirrhotic patients[12]. This result also suggests the hypothesis that BCAA might have an anti-cancer effect on the development of HCC in obese cirrhotic patients. Therefore, in order to verify our hypothesis and clarify the detailed mechanisms of BCAA in the prevention of obesity-related HCC development, we conducted an experimental study by using a DEN-induced HCC model in obese and diabetic *db/db* mice[54]. In this study, BCAA supplementation significantly inhibited the incidence of liver neoplasms, including hepatic adenoma and HCC, through the inhibition of IGF-1, IGF-2, and IGF-1R protein expression in the liver[54]. The reduced incidence of liver neoplasms in this model was associated with improvements in insulin resistance, hyperleptinemia, and liver steatosis[54]. Moreover, our recent publication demonstrated that the administration of BCAA significantly suppressed the spontaneous development of liver neoplasms in *db/db* mice by inhibiting the expression of TNF-α, IL-6, and IL-1β mRNA in the liver[135]. Furthermore, BCAA inhibited the infiltration of macrophages into white adipose tissues, resulting in a reduction of TNF-α and IL-6 mRNA production from the tissue[135]. Thus, BCAA inhibited the development of liver tumorigenesis in obese rodents by regulating various obesity-induced metabolic abnormalities, especially insulin resistance and chronic inflammation, suggesting that BCAA is a promising agent for the chemoprevention of liver carcinogenesis in obese patients (Figure 2).

PREVENTION OF OBESITY-RELATED HCC USING THE PHARMACEUTICAL APPROACH: ACYCLIC RETINOID

Retinoids, derivatives of vitamin A, exert their biological functions by regulating the transcription of target genes through two distinct nuclear receptors - retinoic acid (RA) receptors (RARs) and retinoid X receptors (RXRs), both of which consist of three subtypes (α, β, and γ) characterized by a modular domain structure[136,137]. Among these receptors, RXRα is the most abundant RXR subtype in the adult liver[138]. Once RXRα is activated by its specific ligands, including RA or 9-cis-RA (9cRA), RXRα forms homodimers with itself or heterodimers with other RARs and then interacts with their respective DNA response elements, resulting in the regulation of proliferation, differentiation, and

apoptosis of liver cells. Thus, RXRα plays a crucial role in maintaining the homeostasis of liver cells.

Reduced expression of RXRα has been associated with carcinogen-induced rat hepatocarcinogenesis[139]. The impact of impaired receptor function of RXRα in the development of HCC is demonstrated through experimental studies. In HCC, RXRα is highly phosphorylated by an activated Ras-Erk 1/2 pathway, and accumulates in HCC by preventing its normal degradation through the ubiquitin-proteasome pathway[140]. The accumulated RXRα abrogates the function of the remaining intact RXRα in a dominant-negative manner, thereby inhibiting the formation of heterodimers with the partner molecules, including a tumor suppressor gene, *RAR*β[141-144]. In addition, phosphorylated RXRα is refractory to its potent ligand, 9cRA, and evades 9cRA-induced apoptosis[145]. Thus, the impaired receptor function of RXRα due to phospho-modification also plays a critical role in the development of HCC, suggesting that phosphorylated-RXRα may in the future be a key target for HCC chemoprevention and treatment.

Acyclic retinoid (ACR), which is equivalent to NIK-333 or peretinoin (Kowa Pharmaceutical Co., Tokyo, Japan), is a synthetic retinoid developed for HCC chemoprevention[139]. Recently, the chemopreventive effects of ACR were reported in our clinical studies. A randomized, controlled clinical trial examined the chemopreventive effects of ACR on secondary HCC in patients who underwent curative treatment for initial HCC[146-148]. In this study, oral administration of ACR (n = 44 patients; dose = 600 mg/d) for 12 mo significantly reduced the incidence of post-therapeutic recurrence or new HCC development compared to the placebo group (n = 45 patients) (median follow-up time = 38 mo; P = 0.04)[146,147]. Moreover, the preventative effects of ACR lasted for up to 3 years following the completion of ACR administration[148]. In addition, a subgroup analysis of a large-scale, randomized, placebo-controlled study (n = 401 patients) also showed that ACR (n = 100 patients; dose = 600 mg/d) reduced the risk of HCC recurrence or death by approximately 40% compared to placebo (n = 106 patients), especially in patients with Child-Pugh A and small tumors (size < 20 mm) (P = 0.0347)[149].

The possible molecular mechanisms by which ACR prevents the recurrence and the development of secondary HCC have been elucidated in experimental studies using HCC cell lines. We found that ACR restores the impaired receptor functions of RXRα by inhibiting RXRα phosphorylation. Namely, ACR inhibits the activated Ras-Erk 1/2 pathway independent of RXRα, and consequently prevents phospho-modification of RXRα, thereby restoring the function of RXRα in HCC cells[150]. Furthermore, we found that ACR inhibits not only the Ras-Erk 1/2 pathways but also several types of growth factors and their corresponding RTKs in several malignancies, including HCC[151-156]. Moreover, ACR itself functions as a ligand for RXRα and regulates the expression of its downstream genes such as *p21*, *RAR*β, and Cyclin D1, thereby preventing HCC development through inhibition of cell proliferation or induction of differentiation and apoptosis[145,153,157,158]. Thus, ACR may prevent the development of HCC *via* the pleiotropic responses of ACR target molecules, including phosphorylated RXRα.

Interestingly, our experimental study also elucidated the chemopreventive effects of ACR on the development of obesity-related HCC using the DEN-induced HCC model of obesity and diabetic *db/db* mice[108]. In this study, ACR significantly reduced the incidence of obesity-related HCC by inhibiting Ras activation and the phosphorylation of Erk-1/2 and RXRα, thereby restoring RXRα function in the liver[108]. Notably, the administration of ACR improved obesity-related metabolic abnormalities, such as insulin resistance and liver steatosis[108]. Moreover, ACR treatment decreased the levels of serum TNFα, as well as the expression of TNFα, IL-6, and IL-1β mRNA in the liver, resulting in an improvement of chronic inflammation[108]. As stated above, insulin resistance and chronic inflammation are significant risk factors for the development of obesity-related HCC. Therefore, the use of ACR in obese and cirrhotic patients with diabetes might be an effective strategy in preventing obesity-related HCC (Figure 2).

CONCLUSION

In this review, we highlighted nutraceutical and pharmaceutical approaches to targeting metabolic abnormalities as promising strategies to prevent the development of HCC in obese individuals. The molecular abnormalities represented by (1) insulin resistance/hyperinsulinemia; (2) chronic inflammation; (3) adipokine imbalance; and (4) oxidative stress, are regarded as the likely molecular mechanisms linking obesity to cancer development, including HCC. As stated above, the nutraceutical agents GTCs and BCAA prevented the development of obesity-related HCC by inhibiting those abnormalities in obese and diabetic mice. The safety of both GTCs and BCAA has been shown in recent clinical studies[123-125,127,129,130], suggesting that an intervention using GTCs and BCAA might be a practical approach for the chemoprevention of obesity-related HCC.

In addition, the pharmaceutical agent ACR has chemopreventive effects for recurrence or secondary HCC after curative treatment[146-149], and the safety of the agent was demonstrated in our recent clinical studies[146-149,159]. Moreover, ACR significantly reduced the incidence of obesity-related HCC in obese and diabetic mice, and this effect was associated with an improvement in metabolic abnormalities, including insulin resistance and chronic inflammation[108]. While the detailed mechanism by which ACR improves metabolic homeostasis remains unclear, the positive effects of ACR observed in the experimental study[108]

may encourage the use of the agent for the prevention of HCC in obese individuals.

In conclusion, recent evidence highlights that nutraceutical and pharmaceutical approaches that target metabolic abnormalities are a promising strategy for preventing the development of obesity-related HCC. GTCs, BCAA, and ACR might be candidates for this strategy. Further clinical studies are needed to investigate if active intervention using one or more of these agents can prevent the development of obesity-related HCC.

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REVIEW

Treatment of hepatocellular carcinoma with portal venous tumor thrombosis: A comprehensive review

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Abstract

The natural history of hepatocellular carcinoma (HCC) with portal vein tumor thrombosis (PVTT) is dismal (approximately 2-4 mo), and PVTT is reportedly found in 10%-40% of HCC patients at diagnosis. According to the Barcelona Clinic Liver Cancer (BCLC) Staging System (which is the most widely adopted HCC management guideline), sorafenib is the standard of care for advanced HCC (*i.e.*, BCLC stage C) and the presence of PVTT is included in this category. However, sorafenib treatment only marginally prolongs patient survival and, notably, its therapeutic efficacy is reduced in patients with PVTT. In this context, there have been diverse efforts to develop alternatives to current standard systemic chemotherapies or combination treatment options. To date, many studies on transarterial chemoembolization, 3-dimensional conformal radiotherapy, hepatic arterial chemotherapy, and transarterial radioembolization report better overall survival than sorafenib therapy alone, but their outcomes need to be verified in future prospective, randomized controlled studies in order to be incorporated into current treatment guidelines. Additionally, combination strategies have been applied to treat HCC patients with PVTT, with the hope that the possible synergistic actions among different treatment modalities would provide promising results. This narrative review describes the current status of the management options for HCC with PVTT, with a focus on overall survival.

Key words: Hepatocellular carcinoma; Portal vein tumor thrombosis; Sorafenib; Transarterial chemoembolization; Transarterial radioembolization; Hepatic arterial chemotherapy; Radiotherapy

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Core tip: Hepatocellular carcinoma (HCC) with portal vein tumor thrombosis (PVTT) is associated with a grave prognosis if left untreated. Sorafenib is the only treatment modality recommended for treating HCC patients with PVTT according to most international HCC

treatment guidelines. However, the survival benefits observed following systemic sorafenib treatment are only marginal. Under these circumstances, the need for better treatment options remains unfulfilled. In this comprehensive review, various treatment options are presented-including transarterial chemoembolization, transarterial radioembolization, hepatic arterial infusion, chemotherapy, and radiotherapy-and their outcomes, along with combination strategies.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and the world's fifth most common cancer, and predominantly occurs in patients with liver cirrhosis[1]. Portal vein tumor thrombosis (PVTT) is reportedly found in 10%-40% of HCC patients at diagnosis[2-4]. PVTT is associated with a dismal prognosis, as it is closely related to intrahepatic metastasis and tumor recurrence, and patients only demonstrate 2-4 mo of overall survival with the best supportive care[3-5].

In HCC patients with PVTT, the management options are limited and the optimal treatments remain largely controversial. Curative-intent surgery is often technically challenging, and liver transplant is mostly contraindicated due to high tumor recurrence rates[6,7]. Transarterial chemoembolization (TACE) is generally contraindicated as it can subsequently induce hepatic necrosis and worsen liver function. Radiofrequency ablation is not effective or safe due to the proximity of the hepatic vasculature. External beam radiation plays a limited role in PVTT due to the sensitivity of the liver to radiation and potential for liver failure.

The Barcelona Clinic Liver Cancer (BCLC) Staging System is the most widely adopted HCC management guideline, which classifies HCC with PVTT as advanced HCC (BCLC stage C)[8]. According to the BCLC guidelines, sorafenib is the standard of care for patients with advanced HCC (Figure 1). However, the survival benefits observed following sorafenib treatment are limited, and this underscores the need for better treatment strategies[9]. In recent years, there have been attempts to develop alternative or combination treatments in order to improve the overall survival of patients with HCC and PVTT. In this narrative article, these diverse treatment modalities are thoroughly reviewed (Table 1).

SYSTEMIC THERAPY

Sorafenib-an oral multi-kinase tyrosine inhibitor that demonstrates antiproliferative and antiangiogenic effects-is the only drug proven to improve overall survival in patients with advanced HCC, including those with PVTT[10]. The SHARP trial reported better median overall survival without significant drug toxicity in sorafenib-treated patients (10.7 mo in the sorafenib group *vs* 7.9 mo in placebo group; HR = 0.69; 95%CI: 0.55-0.87, $P < 0.001$)[9]. Subsequent subgroup analyses revealed that sorafenib consistently improved the median overall survival (OS) and median time to tumor progression (TTP) in comparison with the control group, irrespective of disease etiology, baseline tumor extent (*e.g.*, presence of macroscopic vascular invasion), tumor stage (BCLC B-C), prior therapy, or performance status. In particular, patients with macrovascular invasion who were treated with sorafenib demonstrated a longer median OS (8.1 mo *vs* 4.9 mo) and TTP (4.1 mo *vs* 2.7 mo)[10]. Similar findings were also identified in an Asian-Pacific population (6.5 mo in the sorafenib group *vs* 4.2 mo in the placebo group; HR = 0.68; 95%CI: 0.50-0.93; $P = 0.014$)[9,11]. Regarding the safety profile, the most common adverse events included diarrhea, hand-foot skin reaction, fatigue, and skin rash. However, the incidence of serious or life-threatening complications was rare and not affected by the baseline patient characteristics. Therefore, it was suggested that sorafenib could be administered to a wide range of patients with HCC[10]. Since these 2 key studies were released, sorafenib has been considered the standard of care for HCC patients with PVTT by many treatment guidelines such as BCLC Staging System, European Association for the Study of Liver Disease (EASL), and American Association for the Study of Liver Diseases (AASLD)[8,12]. A retrospective study of 30 HCC patients with PVTT demonstrated that the median OS was 3.1 mo and the disease control rate was 33.3%, respectively[13]. Dose reduction was required in 13 patients due to fatigue, hand-foot syndrome, diarrhea, nausea, and skin rash, but no grade 4 adverse events occurred. Interestingly, 3 patients with a partial response achieved marked PVTT revascularization, and responsive patients demonstrated a significantly prolonged OS in comparison with nonresponders. It is assumed that sorafenib exerts antithrombotic effects on PVTT by inhibiting the vascular endothelial growth factor (VEGF) receptor pathway. Therefore, a sensitivity analysis is warranted in order to predict good responders to Sorafenib treatment.

Unfortunately, the observed survival benefits of sorafenib in HCC patients with PVTT are modest, and in recent years there have been efforts to combine systemic chemotherapy with other locoregional therapies in order to improve treatment outcomes.

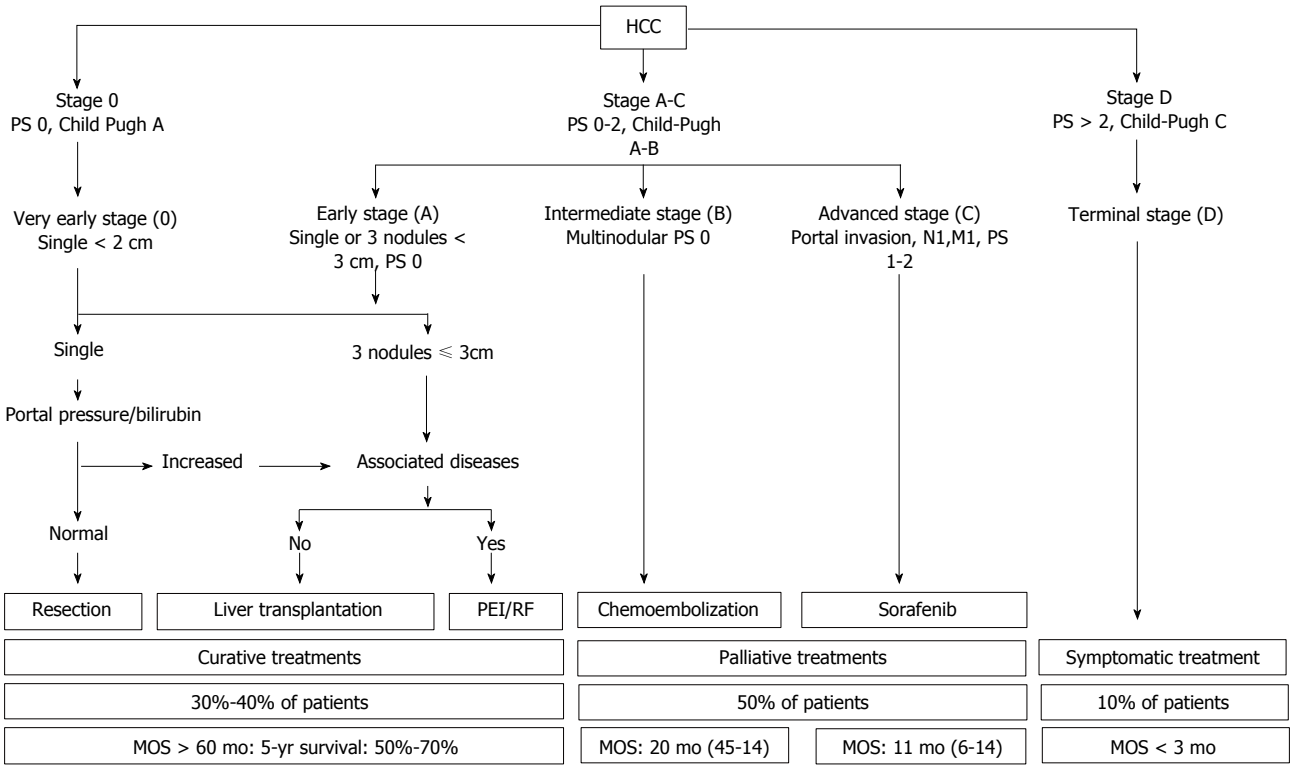

Figure 1 Updated barcelona clinic liver cancer staging system and treatment strategy. HCC: Hepatocellular carcinoma; RT: Radiation therapy.

In addition, there are several other systemic agents under development, but none have demonstrated improved OS.

TRANSARTERIAL CHEMOEMBOLIZATION

Transarterial chemoembolization (TACE) refers to the percutaneous intraarterial introduction of an embolic agent that occludes tumor feeders in combination with an anticancer agent, with the aim of delivering sustained drug levels to the HCC. The anticancer agent is mixed with Lipiodol or loaded onto microspheres. Until now, TACE has been widely used to treat HCC in different stages and plays an established role in the treatment of unresectable HCC[14-18]. In the presence of PVTT, however, TACE is theoretically contraindicated because of the potential risk of hepatic insufficiency that results from ischemia following TACE. However, recent studies demonstrate that TACE can be safely performed in the presence of adequate collateral circulation around the occluded portal vein[19,20].

Chung *et al*[21] have investigated the efficacy and safety of TACE in patients with HCC and main PVTT and reported a median OS period of 3.7 mo. The median survival of the TACE group was significantly longer than the supportive care group (5.6 mo *vs* 2.2 mo). TACE and Child-Pugh A classification were independent predictive factors associated with better overall survival. Regarding complications, no procedure-related deaths were reported within 4 wk after TACE, and morbidity was 28.9%. A prospective comparative study investigated the efficacy and safety of administering TACE to HCC patients with PVTT in comparison with conservative management[22]. In that study, the TACE group demonstrated significantly better OS than the conservative treatment group (7.1 mo *vs* 4.1 mo), and TACE-related complications were adequately managed using conservative treatment. According to the subgroup analysis of segmental and major PVTT, the TACE group also demonstrated significantly better survival. Treatment type, PVTT extent, tumor size, and serum bilirubin were independent prognostic factors of survival on multivariate analysis. A recent meta-analysis, which included the aforementioned study, showed that patients who underwent a TACE procedure demonstrated a significantly better 1-year survival rate in comparison with patients who received conservative treatment (OR = 3.079; 95%CI: 1.094-8.662)[23].

As an alternative to conventional lipiodol-based TACE, nonresorbable microspheres can be loaded with an anticancer agent and intraarterially infused to increase the local drug concentration and reduce systemic toxicity[24]. These particles are known as drug-eluting beads (DEB). There are few studies on using DEB-TACE to treat HCC and PVTT. Kalva *et al*[25] evaluated the safety and efficacy of administering DEB-TACE to advanced HCC patients, including those with lobar PVTT. The median OS was 13.3 mo, and the presence of portal vein thrombosis demonstrated no statistically significant association with OS.

As demonstrated by various studies, TACE is

Table 1 Summary of combination treatments for hepatocellular carcinoma patients with portal vein tumor thrombosis

	Overall survival (mo)	Extent of PVTT (mo)		Ref.
		Main PVTT	Branch PVTT	
BSC	2-4			Llovet *et al*[3], Schöniger-Hekele *et al*[5]
Sorafenib	6.5-8.1			Llovet *et al*[9], Cheng *et al*[11]
TACE	7-10	5.3	10	Chung *et al*[21], Luo *et al*[22]
HAIC	6.5-14			Park *et al*[26], Ando *et al*[27], Eun *et al*[28]
RT	9.6-10.9			Toya *et al*[39], Nakazawa *et al*[40]
TARE	6-16.9	7.7	16.9	Salem *et al*[47], Kulik *et al*[49], Sangro *et al*[48], Memon *et al*[50]
TACE plus sorafenib	11-13	3	13-15	Pan *et al*[58], Zhu *et al*[59]
Sorafenib plus RT	8.6-10.6			Chen *et al*[53], Chow *et al*[61]
TACE plus RT	10.6-12	12		Yoon *et al*[64], Chung *et al*[72], Kim *et al*[73]
HAIC plus RT	12.1			Fujino *et al*[76]

BSC: Best supportive care; TACE: Transarterial chemoembolization; HAIC: Hepatic arterial infusion chemotherapy; RT: Radiation therapy; TARE: Transarterial radioembolization; PVTT: Portal vein thrombosis.

considered safe and feasible for select patients with unresectable HCC, PVTT, and preserved liver function and collateral portal venous circulation. However, to date, the reported OS period for HCC patients with PVTT who receive TACE is slightly better than that of patients who receive sorafenib therapy, though this claim needs to be validated in a prospective study.

HEPATIC ARTERIAL INFUSION CHEMOTHERAPY

Hepatic arterial infusion chemotherapy (HAIC) using an implantable port system has been applied to treat advanced HCC with PVTT. HAIC is theoretically more effective against HCC than systemic chemotherapy because it provides the direct delivery of a high concentration of the anticancer agent to the tumor through the hepatic artery. HAIC also minimizes systemic toxicities due to first-pass effects[26]. HAIC is usually administered using 1 of the following 3 well-reported regimens: cisplatin alone, 5-FU plus cisplatin, or 5-FU plus interferon. Many studies have been conducted on advanced HCC with PVTT and demonstrate a median OS of 6.5-14 mo[26-29]. Quite recently, Song *et al*[30] conducted a multicenter study to compare the efficacy of sorafenib with HAIC in HCC patients with PVTT. In their study, the median OS (7.1 mo *vs* 5.5 mo, P = 0.011) and TTP were significantly longer in the HAIC group than in the sorafenib group (3.3 mo *vs* 2.2 mo, P = 0.034).

Regarding the safety profile of HAIC, hematologic complications (*e.g.*, anemia, neutropenia, thrombocytopenia) and gastrointestinal toxicity (*e.g.*, nausea, vomiting, abdominal pain) can occur. Most HAIC-related toxicities are transient, tolerable, and successfully controlled with conservative treatment, although some patients end up withdrawing from HAIC therapy. In addition, catheter-related complications (*e.g.*, hematoma, catheter occlusion, infection) can also occur[31,32]. However, HAIC is not recommended as a standard treatment for HCC patients with PVTT, as these data are mostly from Japan and there is a lack of randomized controlled trials. To become an alternative to current Sorafenib treatment, the better outcomes observed in previous retrospective studies using HAIC need to be verified by future prospective studies and validated in a Western population.

EXTERNAL BEAM RADIATION

The role of external beam radiation therapy (RT) for HCC is limited due to the risks of radiation-induced liver disease and the low tolerance of the whole liver to RT[33]. However, rapid advances in radiotherapy techniques, including 3-dimensional conformal radiotherapy and image-guided radiotherapy, as well as knowledge on partial volume liver tolerance, have enabled the delivery of higher radiation doses to HCC than in the past, thereby allowing RT to be used as a potential standalone or adjunct treatment for HCC[34-36].

Notably, one of the primary indications for RT is the presence of PVTT, and previous studies report good treatment responses and promising outcomes using RT[37,38]. A retrospective study evaluated the treatment outcomes of RT in 38 patients with HCC with PVTT[39]. In that study, the treatment rate was 44.7% and OS was 9.6 mo. Nakazawa *et al*[40] recently compared standard sorafenib therapy to RT in patients with unresectable HCC with main or first-branch PVTT, and they reported a longer median OS in the RT group (10.9 mo *vs* 4.8 mo, P = 0.025) after performing propensity score analysis (28 pairs). In their study, whereas almost half the patients discontinued sorafenib due to adverse events, there was no grade 3 or higher gastrointestinal or hepatic toxicity and grade 3 leukocytopenia was only observed in 1 patient in the RT group. Because HAIC is not regarded as a standard therapeutic modality, future large-scale and prospective studies are warranted in order to test the clinical efficacy and safety of using RT to treat HCC with PVTT.

TRANSARTERIAL RADIOEMBOLIZATION

Transarterial radioembolization (TARE) and selective

internal radiation therapy (SIRT) using beta-emitting yittrium-90 in resin microspheres or glass particles have been introduced as alternatives to TACE for HCC[41]. TARE differs from TACE in that it offers antitumor effects in the form of local beta radiation, not arterial obstruction. The embolic materials are loaded with yittrium-90 and administered *via* intraarterial injection, which allows lobar, segmental, and subsegmental therapy. The average penetration depth by this local radiation into the liver tissue is approximately 2.5 mm, thus sparing the normal adjacent liver from damage and obviating the need of postprocedure isolation. The half-life is 64 h, and almost the entire radiation dose is delivered within 14 d of the procedure[42-44]. There is some evidence that TARE results in encouraging outcomes, especially in patients with PVTT which is a contraindication to TACE[8,45,46]. Administering TARE to patients with advanced HCC has demonstrated a median OS of 6-10 mo, which is comparable to the 6.5-10.7 mo reported in landmark studies on sorafenib[9,11]. Because it produces much fewer embolic effects than TACE, PVTT is not a contraindication for TARE, but the presence and extent of PVTT does affect prognosis. Salem *et al*[47] studied the long-term outcomes of using TARE to treat HCC and reported a median OS of 16.6 mo in Child-Pugh A patients with branch PVTT *vs* 6.5 mo in Child-Pugh B patients. Among patients with main PVTT, the median OS decreased to 7.7 mo in Child-Pugh A patients and 4.5 mo in Child-Pugh B patients. Smaller studies using TARE report concordant results. When the distinction between main and branch PVTT was made, the median survival periods reported by Sangro *et al*[48] were 9.7 and 10.7 mo, and those reported by Kulik *et al*[49] were 4.4 and 9.9 mo. Memon *et al*[50] reported even higher median survival periods of 15.7 and 9 mo in Child-Pugh A patients, respectively. Mazzaferro *et al*[51] performed a single-center, prospective, phase Ⅱ trial to study the efficacy of TACE on HCC patients with PVTT. They reported a median OS of 13 mo in 35 HCC patients with branch or main PVTT and better patient survival in Child-Pugh A patients (16 mo *vs* 6 mo). In a recent retrospective study, Gramenzi *et al*[52] compared the outcomes of sorafenib and TARE in patients with advanced HCC. The median OS values of the 2 groups were comparable: 13.2 mo in the TARE group (63 patients) and 14.4 mo in the sorafenib group (74 patients). Following propensity score analysis (38 pairs), the median OS did not differ between groups. Ongoing randomized controlled trials that compare standard sorafenib therapy to TARE as a first- or second-line treatment for HCC patients with PVTT are expected to define the populations that benefit from this therapeutic modality.

TARE is generally well tolerated, and the most common complication is postembolization syndrome which occurs in 20%-55% of patients. Postembolization syndrome consists of various symptoms (*e.g.*, fatigue, fever, nausea, vomiting, abdominal pain), which are usually well-tolerated with conservative management. Other reported complications, such as radiation-induced liver disease, radiation pneumonitis, radiation cholecystitis, biloma, hepatic abscess, and biliary stricture, are uncommon[47,49]. TARE can lead to severe adverse events, such as gastrointestinal ulcerations, in < 5% of patients[53,54]. However, gastrointestinal toxicities may be prevented by carefully administering preemptive coil embolization.

EMERGENCE OF COMBINATION STRATEGIES FOR TREATING HCC WITH PVTT

As mentioned earlier, the current standard sorafenib treatment only provides modest survival benefits, and, thus, investigators have made concerted efforts to combine different modalities, which will be discussed in the following sections.

TACE IN COMBINATION WITH SORAFENIB

The efficacy of sorafenib in combination with TACE has been investigated, as these two therapeutic options are expected to work synergistically. TACE-induced hypoxia in surviving tumor cells results in the release of angiogenic growth factors, which contribute to tumor recurrence, metastasis, and worse outcomes[55,56]. Sorafenib suppresses tumor cell proliferation by exerting antiangiogenic effects through the blocking of VEGF receptor-2 and -3 and platelet-derived growth factor receptor tyrosine kinase[57]. A retrospective study on combining TACE and sorafenib to treat HCC patients with PVTT (branch and main PVTT) demonstrated a median OS of 13 mo and median TTP of 7 mo, respectively[58]. Procedure-related mortality and grade 4 adverse events did not occur. Child-Pugh class, extrahepatic metastasis, and gross morphologic type were prognostic factors. Zhu *et al*[59] compared the outcomes of sorafenib plus TACE to the outcomes of TACE alone in patients with HCC and PVTT. TACE plus sorafenib demonstrated significant survival benefits in comparison with TACE alone (11 mo *vs* 6 mo, $P < 0.001$). When considering first-, second-, and lower-order branch PVTT, subgroup analyses of OS in patients with different types of PVTT revealed that the median OS of patients treated with TACE plus sorafenib is significantly longer than that of patients treated with TACE alone (13 mo *vs* 6 mo for patients with first-order PVTT; 15 mo *vs* 10 mo for patients with second- or lower-order PVTT). In patients with main PVTT, no survival benefit was observed between groups (3 mo in both groups). The worsening of liver function after TACE-sorafenib treatment was only noted in patients with main PVTT, and sorafenib-related complications classified as grade 3 or higher

occurred in 16 patients (35%).

A phase Ⅱ prospective trial (START trial) is under way in which the efficacy of TACE plus sorafenib when administered to HCC patients (including those with branch PVTT) will be investigated. Interim analysis has indicated promising outcomes and acceptable adverse event rates, and, thus, it is expected that the role of combination therapy in HCC patients with PVTT will be determined within the foreseeable future.

SORAFENIB PLUS RADIOTHERAPY

The combination of sorafenib and radiotherapy is based on the finding that sorafenib enhances the radiosensitivity of human HCC cell lines by selectively inhibiting the radiation-induced activation of the VEGFR2 and extracellular signal-regulated kinase (ERK) pathways, thereby promoting radiation-induced apoptosis[60]. In this context, the concurrent administration of sorafenib and radiotherapy-in the form of either TARE or external beam radiation-is expected to work synergistically on advanced HCC. In a phase Ⅱ study on combining sorafenib and external beam radiation therapy, the mean OS was 10.6 mo in Child-Pugh A patients with PVTT. Of the 40 patients analyzed, 4 (10%) and 6 patients (15%) experienced grade 3 or higher hepatic toxicities during or before RT, respectively. Therefore, special care needs to be taken when combination therapy is considered[53]. Combination of sorafenib and TARE therapy is reportedly well-tolerated, and the mean OS was reported to be 8.6 mo in advanced HCC patients[61]. However, that study only included patients with branch PVTT, not major PVTT. In a recent prospective study on the safety profile of combination TARE-sorafenib treatment, the incidences of total and > grade 3 adverse events did not statistically differ between the combination treatment and sorafenib-alone groups[62].

TACE PLUS RADIOTHERAPY

TACE in combination with radiotherapy is a newly introduced combination strategy that results in improved outcomes in HCC patients with PVTT[38,39,63-70]. The rationale behind this combined treatment is that reducing PVTT with radiotherapy may inhibit intravascular tumor growth and preserve adequate portal venous flow, thereby preventing the deterioration of liver function, limiting intrahepatic tumor spread, and facilitating subsequent treatments for the primary tumor[66,68]. In addition, radiotherapy may potentially increase the effects of subsequent chemoembolization by inducing the regression of the arterioportal shunt around the PVTT[71].

In a recent retrospective study, Chung *et al*[72] evaluated the safety and survival outcomes of TACE plus radiotherapy in patients with HCC invading the main portal vein. After chemoembolization, major complications occurred in 30 of 151 patients (19.9%) and were more frequently seen in Child-Pugh B patients. The 30-d mortality rate was 0.7%, and most adverse events were managed by conservative treatment. In addition, adjuvant RT for main PVTT after chemoembolization in 147 patients was uneventful without RT-associated adverse events. The median OS period was 12 mo (14 mo in Child-Pugh class A patients *vs* 8 mo in Child-Pugh class B patients). Yoon *et al*[64] also studied the efficacy of TACE in combination with RT for HCC with PVTT (main or bilateral/unilateral) and reported a 28.1% tumor response rate and OS of 10.6 mo. Kim *et al*[73] compared the efficacy of TACE with or without RT *vs* sorafenib for advanced HCC with PVTT. In that study, patients were divided into 3 different treatment pairs (TACE *vs* TACE + RT; TACE *vs* sorafenib; and TACE + RT *vs* sorafenib). According to the propensity score matched analysis, the group that received TACE in combination with radiotherapy demonstrated longer TTP and OS values than the groups that received TACE alone (102 pairs; 8.7 mo *vs* 3.6 mo, $P < 0.01$; 11.4 mo *vs* 7.4 mo, $P = 0.023$, respectively) or sorafenib-alone groups (30 pairs; 3.4 mo *vs* 1.8 mo, $P < 0.01$; 5.9 mo *vs* 4.4 mo, $P = 0.03$, respectively). Although these outcomes need to be verified by future randomized studies, TACE in combination with radiotherapy could serve as an alternative to current standard sorafenib therapy.

HEPATIC ARTERIAL INFUSION AND RADIOTHERAPY

Some investigators have combined HAIC with conformal radiotherapy to treat HCC patients with PVTT and reported the efficacy of arterial infusion chemotherapy plus radiotherapy[74,75]. In a recent retrospective study, Fujino *et al*[76] investigated the efficacy of combination therapy for major or first-order branch PVTT and reported that the combination group demonstrated a significantly longer median OS (12.1 mo *vs* 7.2 mo) and higher objective response rate than the HAIC-alone group when used to treat intrahepatic HCC patients who were nonresponsive to HAIC (objective response rate = 56.1% *vs* 33.3%; median OS = 8.6 mo *vs* 5 mo), but no significant differences were noted in intrahepatic responders. It is noteworthy that reducing PVTT volume with radiotherapy may help patients respond better to HAIC.

OTHER COMBINATION STRATEGIES

Various novel treatment modalities have been developed in an effort to reduce PVTT burden. According to the BCLC Staging System, radiofrequency ablation (RFA) is the standard care for early, stage A HCC. Recently, however, it was reported that RFA may improve patient survival and has come into use as a treatment modality for HCC patients with PVTT[77,78].

TACE in combination with RFA also confers survival benefits to PVTT patients[79]. Iodine-125 seeds have been used to treat solid tumors, and their use in HCC patients with PVTT is reportedly safe and feasible[80]. A recent prospective study compared the efficacy and safety of TACE in combination with the endovascular implantation of an iodine-125 seed strand for PVTT *vs* TACE alone. In that study, TACE in combination with iodine-125 seeds demonstrated better median OS than TACE alone[81].

GENERAL TREATMENT RECOMMENDATIONS FOR HCC WITH PVTT

Although the BCLC system, which is based on data from randomized controlled studies, has been widely validated, it does not reflect the diverse situations that present in clinical practice. In particular, because advanced HCC (*i.e.*, BCLC C stage) affects heterogeneous patient populations, different treatment modalities or combination therapies have been advocated in order to obtain better treatment outcomes. In HCC patients with PVTT, the marginal survival benefits observed with sorafenib may be attributed to patient heterogeneity. Therefore, subclassification of BCLC stage C patients is thought to be the first step to providing more individualized treatments to patients with this stage of cancer. Under these circumstances, the recently introduced Hong Kong Liver Cancer Staging System subdivides macrovascular invasion into intrahepatic and extrahepatic vascular invasions and recommends administering more aggressive treatment to early- and intermediate-stage cancers[82]. Regarding the various therapeutic modalities reviewed above, it is not easy to reach a consensus regarding the best treatment options for individual patients and remains an area of active discussion because the data on each modality reflects regional differences in patient characteristics and clinical practice. In particular, the ongoing observational study (GIDEON) is expected to generate better understanding of the effectiveness and safety of the diverse treatment options for HCC patients with PVTT[83-85].

CONCLUSION

According to the BCLC Staging System, systemic therapy using sorafenib is considered the standard of care for patients with HCC and PVTT despite its modest survival benefits. Other treatment modalities for HCC with PVTT have continued to evolve in recent years (*e.g.*, TACE, HAIC, TARE, radiotherapy, and various combination strategies), and the BCLC recommendations now seem very limiting. However, because there are few phase Ⅰ or Ⅱ studies on multimodal treatments, it is difficult to validate the findings of previous, retrospective, observational studies and thus to reach a consensus regarding the best options for advanced HCC with PVTT. Therefore, future prospective, randomized, controlled studies are warranted to compare the outcomes of diverse treatment modalities, and the observed findings may need to be incorporated into international guidelines.

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Nuclear magnetic resonance based metabolomics and liver diseases: Recent advances and future clinical applications

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Abstract

Metabolomics is defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification. It is an "omics" technique that is situated downstream of genomics, transcriptomics and proteomics. Metabolomics is recognized as a promising technique in the field of systems biology for the evaluation of global metabolic changes. During the last decade, metabolomics approaches have become widely used in the study of liver diseases for the detection of early biomarkers and altered metabolic pathways. It is a powerful technique to improve our pathophysiological knowledge of various liver diseases. It can be a useful tool to help clinicians in the diagnostic process especially to distinguish malignant and non-malignant liver disease as well as to determine the etiology or severity of the liver disease. It can also assess therapeutic response or predict drug induced liver injury. Nevertheless, the usefulness of metabolomics is often not understood by clinicians, especially the concept of metabolomics profiling or fingerprinting. In the present work, after a concise description of the different techniques and processes used in metabolomics, we will review the main research on this subject by focusing specifically on *in vitro* proton nuclear magnetic resonance spectroscopy based metabolomics approaches in human studies. We will first consider the clinical point of view enlighten physicians on this new approach and emphasis its future use in clinical "routine".

Key words: Metabolomics; *In vitro* nuclear magnetic resonance spectroscopy; Liver diseases; Cirrhosis

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Core tip: Metabolomics is a powerful technique to improve our pathophysiological knowledge of various liver diseases, to help clinicians in the diagnostic process as well as in the prognosis or therapeutic response assessment. Nevertheless, the usefulness of metabolomics is often not understood by clinicians. In the present work, after a concise description of the different techniques and processes used in metabolomics, we will review the main research on this subject by focusing specifically on proton nuclear magnetic resonance based metabolomics in human studies. Three major themes will be enlightened: acute liver disease, chronic liver disease and liver transplantation.

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INTRODUCTION

Metabolomics is an "omics" technique that is situated downstream of genomics, transcriptomics and proteomics (Figure 1)[1]. Metabolomics is the study (and by the way the monitoring) of metabolic changes in an integrated biological system related to (patho-) physiological stimuli or genetic modification, using multiparametric analyses.

The fundamental of metabolomics is that disease (or therapeutic response) can be reflected by changes in metabolite concentrations in biological fluids or tissues. This concept is not really new. More than 500 years ago, the "urine wheel" was published by Ullrich Pinder in his book (the *Epiphanie Medicorum*, 1506*)*. The wheel described color, taste and smell of urine to help physicians in making-diagnoses. In other words, the phenotype of the urine was used to help the clinician. Now analytical tools to measure small-molecule metabolites called biomarkers have become tools in hospital laboratories to assist physicians when diagnosing disease.

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and mass spectroscopy (MS) based metabolomics approaches are the two most applied experimental methods in this area[2]. They allow identifing and quantifing metabolites within a biological fluid (serum, plasma, urine, ascites, bile…) in a single experiment. Metabolites are small molecules (less than 1 kDa) that participate in general metabolic reactions or pathways in biofluid or tissue. The term metabolome, derived from the word genome, refers to the complete set of metabolites in a biofluid, cell, tissue or organism[3]. For example, the human serum metabolome is composed of around 4200 metabolites, half of which are phospholipids and over a thousand glycerolipids (triglycerides, diglycerides, and monoacylglycerols). Amino acids, peptides, carbohydrates, amines, and carboxylic acids are other metabolites being part of serum metabolome[4].

Data analysis in metabolomics and the process between, patients biofluid or tissue collection and the final interpretation have been well standardized[5]. The principal steps to achieve this approach are: collection of fluid or tissues, sample conservation, sample preparation before signal acquisition, signal acquisition in NMR (or MS) platform, data processing and analysis (Figure 2).

Technically, each approach is grounded in different theoretical principles. MS spectroscopy is based on the separation of the metabolites using chemical and physical properties and NMR is based on the magnetic properties of some atoms. In MS spectroscopy, after specific preparation in function of the kind of metabolite analyzed (lipids or amino-acid or other type of metabolite), the sample is ionized and different metabolites are separated by their charges and mass.

NMR spectroscopy is often easier to understand for the physician because using exactly the same principle than magnetic resonance imaging (MRI) that is largely used daily in medicine. The principle behind NMR is that many nuclei have a nuclear spin value (for example: hydrogen, ^{31}phosphorus or ^{13}carbon) and all nuclei are electrically charged. If an external magnetic field is applied, transitions are possible between the ground and excited spin states (*i.e.*, energy transfert between the baseline to a higher energy level). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum. Then instead to obtain an image like in MRI, in ^{1}H NMR spectroscopy the signal is transformed in one or two dimensions spectra (Figure 3).

In ^{1}H NMR spectroscopy, metabolites are identified by peaks shape, peaks multiplicity and peaks correlations. Large databases as the Human Metabolome Database (HMDB) are available to find out all the metabolite characteristics[6,7].

Those techniques have advantages and disadvantages but are often complementary (Table 1).

Basically, ^{1}H NMR metabolomics approach has the advantage of efficiently obtaining information on large numbers of metabolites in biofluids *in vitro* as well as in various tissues *ex vivo* (using HR-MAS:

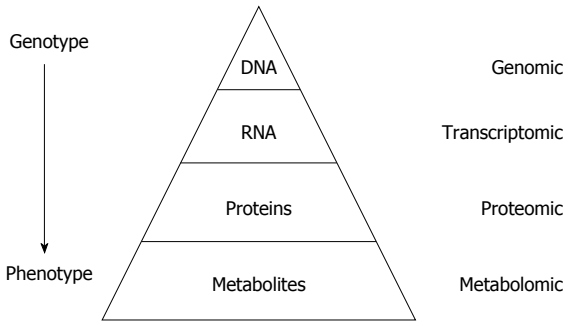

Figure 1 "Omic" techniques. Schematic representation in biological system: Each functional level from the DNA, RNAs, Proteins and metabolites who constitute respectively the genome, transcriptome, proteome and metabolome, have bidirectional flow of information and complex interactions together and with the environment (diseases, drug, lifestyle, genre, habit, diet, *etc.*). Those interactions produce the phenotype that represents the final output of the system measured in metabolomics.

high-resolution magic angle spinning) and *in vivo* (using Medical Magnetic Resonance Imaging). ^{1}H NMR is a highly reproducible, rapid and non-destructive technique. Analysis can be performed with a minimal of sample preparation. Its sensitivity is far lower (microgram *vs* picogram) than mass spectrometry but sufficient to detect most of changes in biological fluid. MS needs more sample preparation and is a destructive technique. Well-conducted reviews provide larges understanding details on the different platforms especially concerning the technical aspect, physical based-mechanism and analytical method use in metabolomics[1,3,8].

Thus these approaches offer high-throughput analyses at relatively low cost per sample. Multi-parameter datasets contain huge quantity of information concerning metabolites in the form of complex spectrum data. Many computer-based processing and statistical tools have been developed to facilitate analysis and interpretation of the data. Statistical models applied to metabolomics data can determine biomarkers or metabolomics profiles[9]. They may help identify biomarkers or the metabolic profile that characterize a disease, and/or evaluate metabolic modifications after treatment has been initiated[3]. The metabolite analysis provides information on the "metabolic phenotype" of the patient in response to various endogenous or exogenous stimuli. Then that may contribute to a better understanding of pathophysiology, to aid diagnosis, in decision-making process in choosing therapy or predicting outcome of therapeutic intervention. In the other hand, in the near future, these tools permit us to give our patients a more personalized approach in medicine[10].

Metabolomics approach showed sometime better diagnosis performance than usual biomarkers. Nevertheless, at the beginning, metabolomics approach should be considered more than complementary tool, helping clinician to diagnose disease, evaluate disease stage or follow treatment that substitution technic.

This review focuses on the recent human metabolomics data in the field of liver disease and their complications using *in vitro* ^{1}H NMR metabolomics approaches. MS based metabolomics approaches and in vivo MR studies are excluded from this review.

APPLICATIONS OF ^{1}H NMR METABOLOMICS APPROACHES IN LIVER DISEASES

The liver is a major organ with several intensive metabolic activities. The metabolic activities are distributed in different zones of the liver parenchyma. Hepatocytes metabolism varies according to its position in the liver: for example, oxidative phosphorylation, glucose output, urea synthesis, and bile acid synthesis is higher in the periportal area, whereas glucose uptake, glutamine formation, and xenobiotic metabolism are greater in the perivenous area[11]. Acute, chronic, and acute-on-chronic conditions perturb regulation of liver metabolism. From this point of view, the blood or urine metabolome should represent the final outcome of liver cellular regulation at many different levels, and for this reason represent the phenotype of a disease or a therapeutic response.

We divided the following parts into three domains: acute liver disease, chronic liver disease and liver transplantation. Major studies and biomarkers examples for each part are shown in Table 2.

Acute liver disease

Drug induced liver injury: Metabolomics have been used since decennia in toxicological studies principally in animal models[12]. Some recent studies have shown that metabolomics can be a powerful tool in human toxicology. Clayton *et al*[13] have used NMR metabolomics to study the secondary metabolism of acetaminophen in urine. The goal was to identify urine biomarkers that might be predictive of the manner in which that individual metabolizes the acetaminophen. In this study, results suggested a mechanism whereby p-cresol and acetaminophen would compete for sulfation. Then higher p-cresol levels would lead to lower levels of sulfated acetaminophen and increase toxicity. More interestingly metabolome analysis have been in evidence the interplay between gut bacteria and secondary drug metabolism because p-cresol is a product of bacterial metabolism of tyrosine in the gut. Winnike *et al*[14] used a pharmaco-metabolomics approach to predict acetaminophen induced liver injury with a therapeutic dose (4 g per day for 7 d). In human healthy volunteers and earlier after the beginning of the treatment, they were able to identify patterns of urinary metabolites that distinguished subjects susceptible to acetaminophen-hepatotoxicity from those who were not susceptible.

Those examples represent a major advance in

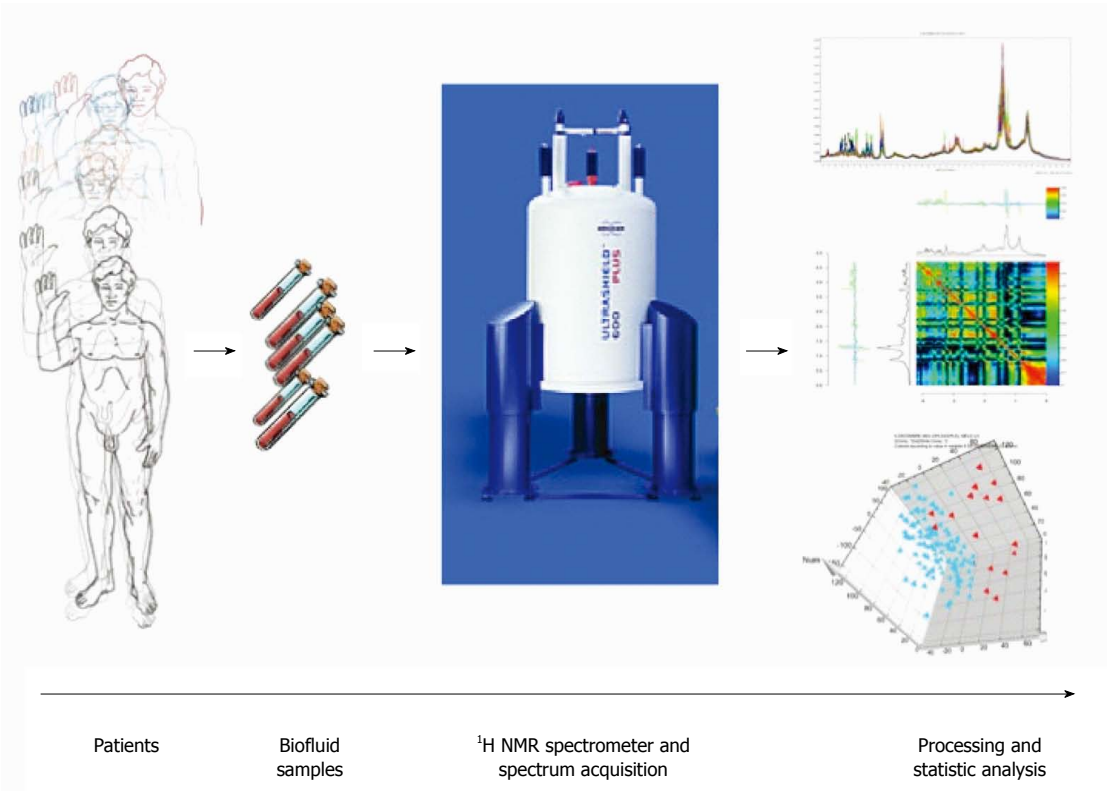

Figure 2 Schematic view of workflow for metabolomic studies: From bedside to bench. Proposed standards for metabolomic approach are presented in this schematic view. Clinical question, selection of the population, standardized biofluid collection and conservation, biofluid preparation and spectra acquisition, pre-processing to clean the data for data processing, pre-treatment (*i.e.*, scaling, centering, *etc.*) to transform the clean data to make them ready for data processing, data analysis (multivariate analysis, unsupervised and supervised analysis), metabolite identification and interpretation.

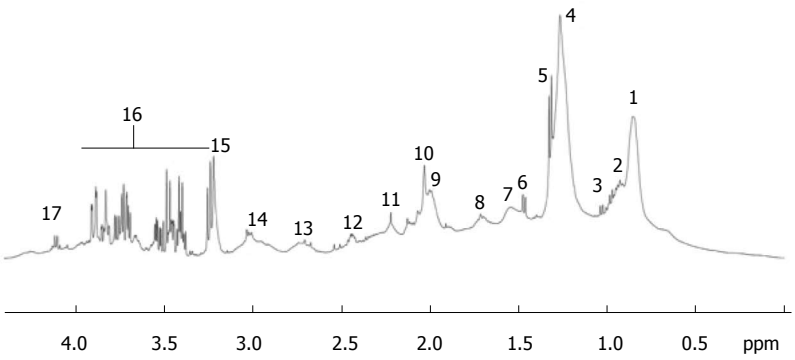

Figure 3 Typical proton nuclear magnetic resonance (500 MHz) spectra of the region between 0 and 6 ppm from cirrhotic patient. Region between 4.5 and 5.0 ppm corresponding to the water and urea was suppressed. Peak assignment: 1: Fatty acids (-CH_2-CH_2-CH_2-CH_3); 2: Isoleucine; 3: Valine; 4: Fatty acids (-CH_2-CH_2-CH_2-); 5: Lactate; 6: Alanine; 7: Fatty acids (-CH_2-CH_2-CO-); 8: Fatty acids (-CH_2-CH_2-CH=); 9: Fatty acids (=CH-CH_2-CH_2-); 10: Acetyl signals from α1-acid glycoprotein; 11: Fatty acids (-CH_2-CO-); 12: Glutamine; 13: Fatty acids (=CH-CH_2-CH=); 14: Albumin lysyl; 15: Choline; 16: Glucose; 17: Lactate; 18: Fatty acids (-CH=CH-). Adapted from Nahon *et al*[29].

personalized medicine. A NMR based metabolomics approach could be a practical method for identifying susceptible patients shortly after starting drug treatment and with high risk of developing DILI.

Liver injury and liver failure: Acute hepatic liver failure is a very severe condition with a high risk of mortality. Predictability of survival without liver transplantation is often difficult. Using ^{1}H NMR on serum and urine, Saxena *et al*[15] has shown the potential to predict an unfavorable outcome from a metabolomic profile-evaluation in patients with fulminant hepatic failure. In this study, metabolomics profiles of patients with Fulminant Hepatic failure and favorable (n = 20) or unfavorable (n = 10) outcomes were compared. The results of this pilot study showed that one single biomarker (Glutamine) could permit to predict unfavorable outcome with a high sensitivity.

In another area of acute liver injury, Ranjan *et al*[16] examined serum in patients of liver injury to explore

Table 1 Advantages and disadvantages of nuclear magnetic resonance and mass spectroscopy

	NMR	MS
Sensitivity (detection limit)	Usually micromolar (nanomolar with cryosonde)	Picomolar
Reproducibility	High	Low
Detected	Non targeted approach	Targeted approach
Metabolite	Detect metabolite Only if contain proton on the molecule	Need specific preparation to well detected some metabolites (Lipids…)
Metabolite identification	Easy, using 1D and/or 2D spectra and databases	More difficult, need sometime complementary analysis
Number of know identifiable metabolites	More than 200	More than 4000
Sample	Simple preparation (minimal add of D_2O, Buffer and sometime reference)	Preparation more complex (protein extraction, *etc.*)
	Non destructive method	Destructive method
	Need 400 μL (less than 10 μL with microprobe)	Need few microliters
Type of sample	Liquid (urine, whole blood, serum, plasma, *etc.*) and intact tissue	Liquid
Cost of machine	Very high	High
Cost of sample analysis	Lower	Higher
Signal acquisition time	5 to 15 min for 1D spectra More longer for 2D spectra (few hours)	Around 10 min

NMR: Nuclear magnetic resonance; MS: Mass spectroscopy.

the possible clinical application of new metabolites as a biomarker to detect traumatic liver damage in blunt abdominal trauma. In this study, ^{1}H NMR spectroscopy revealed only two amino acids, phenylalanine and tyrosine, in the sera of the subjects with liver injury, irrespective of the extent and type of injury gauged by radiology or laparotomy. The author concluded that these biomarkers detected by NMR spectroscopy could get a relevant clinical importance in establishing liver injury in patients with blunt abdominal trauma.

AutoImmune hepatitis: Diagnosis of autoimmune hepatitis (AIH) is often difficult because of lack of specificity of biological and clinical signs. Moreover, diagnosis of AIH might be confounded with other acute liver conditions like DILI or primary biliary cirrhosis or overload from other diseases. Wang *et al*[17] assessed the utility of metabolomics in the diagnosis of the disease. Plasma metabolomics profiles of patients with AIH, PBC, DILI, PBC and healthy subjects were compared. Nine biomarkers showed great sensitivity in discriminating AIH from other disease were identified. The utility for the diagnosis of the biomarker panel was assessed and they achieved good sensitivity and specificity in distinguishing AIH from other diseases.

Chronic liver disease

Chronic viral hepatitis: Different viruses cause hepatitis infection. Hepatitis C virus is one of the most important and has infected about 150 million people worldwide[18]. Expensive biological methods to diagnosis HVC infection are problematic, especially in developing countries.

Several studies using metabolomics approaches in the field of viral hepatitis have been done. One of the most relevant is the study ran by Godoy *et al*[19]. In a metabolomics model based on ^{1}H NMR spectra of urine they discriminated patients with HCV infection with high sensitivity (94%), specificity (97%) and accuracy (95%). These findings reveal the potential of metabolomics as a low cost, noninvasive diagnosis of HCV related hepatitis using urine samples.

Assessment of cirrhosis and its complications: Studies have shown a close relationship between metabolic abnormalities and the severity of the disease in sera and tissues[20-23]. In those studies, a metabolomics approach was a powerful tool in assessing the severity of chronic liver failure in alcohol-induced cirrhosis within a cohort of patients without acute decompensation. For instance, in our previous study, the severity of chronic liver failure was evaluated using the MELD score, and correlated well with impairment of lipid, glucose, and amino acid metabolism[20]. Other studies show that metabolomics can fingerprint the differences between compensated and decompensated cirrhosis, and between cirrhosis caused by alcohol or viruses[24,25].

Assessments of complication in patients with chronic liver disease, especially with cirrhosis, were performed using metabolomics approaches.

Hepatic encephalopathy (HE) is a well-known complication of liver cirrhosis. Its diagnosis is currently performed at the bedside using clinical examination. Unfortunately low grade or "sub-clinical" HE (also called minimal hepatic encephalopathy) is more difficult to diagnose and request using more complex psychometric tests that are time consuming and not realized in routine. In this case, biomarkers could be easier to use and helpful. Jiménez *et al*[26] have shown that using ^{1}H-NMR to assess the metabolomics of sera from patients with cirrhosis could discriminate between patients with minimal hepatic encephalopathy and those with no encephalopathy.

Recently, we described a serum metabolite fingerprint for acute-on-chronic liver failure, obtained

Table 2 Examples of metabolite changes involved in liver diseases in nuclear magnetic resonance based metabolomics approaches

Metabolites	Variation	Model/pathology	Sample	Ref.
2OH butyrate	-	HBV *vs* HEV	Plasma	[36]
3OH butyrate	+	ximelagatran toxicity	Plasma	[37]
3OH butyrate	+	Cirrhosis (HBV/alcohol) *vs* controls	Serum	[24]
Acetate	+	acetaminophen toxicity	Urine	[14]
Acetate	+	HCC *vs* cirrhosis	Serum	[29]
Acetate	-	Cirrhosis severity	Serum	[20]
Acetoacetate	-	Cirrhosis and encephalopathy	Serum	[26]
Acetoacetate	+	Cirrhosis (HBV/alcohol) *vs* controls	Serum	[24]
Acetoacetate	-	HBV *vs* alcohol cirrhosis	Serum	[24]
Acetoacetate	-	HBV *vs* control	Urine	[36]
Acetoacetate	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Acetone	-	HEV *vs* control	Plasma	[36]
Acetone	+	Cirrhosis (HBV/alcohol) *vs* controls	Serum	[24]
Acetone	-	Decompensated *vs* compensated cirrhosis	Serum	[25]
acetone	-	HCC *vs* Cirrhosis *vs* controls	Urine	[31]
Acetone	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
bile acids	+	Cholangicarcinoma *vs* other causes	Bile	[38]
Bile acids	+	HCC *vs* adjacent tissue	Liver tissue	[39]
Carnitine	-	HBV *vs* control	Plasma	[36]
Citrate	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Choline	-	fibrosis *vs* cirrhosis	liver tissue	[21]
Choline	+	HCC *vs* adjacent tissue	liver tissue	[39]
Choline, P-choline	-	Cirrhosis severity	serum	[20]
Creatine	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Creatine	+	high grade HCC *vs* low grade HCC	Liver tissue	[39]
Dimethylamine	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Fatty acids	-	Biliary tract cancer	Bile	[33]
Fatty acids	-	Non fonctionnal *vs* fonctionnal graft after liver transplantation	Blood (extraction)	[34]
fatty acids	-	Cirrhosis and encephalopathy	Serum	[26]
fatty acids (HDL)	-	Cirrhosis severity	Serum	[20]
fatty acids (HDL)	-	HCC *vs* cirrhosis	Serum	[29]
Glutamine	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Glycerol	+	HEV *vs* control	Plasma	[36]
Glycerol	+	Cirrhosis and encephalopathy	Serum	[26]
GPC	-	mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
GPC	+	HCC *vs* adjacent tissue	Liver tissue	[39]
GPC/P-choline	-	Cirrhosis (HBV/alcohol) *vs* controls	Serum	[24]
Histidine	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Isobutyrate	+	Cirrhosis (HBV/alcohol) *vs* controls	Serum	[24]
Isobutyrate	+	HBV *vs* alcohol cirrhosis	Serum	[24]
LDL	-	Cirrhosis (HBV/alcohol) *vs* controls	Serum	[24]
LDL	-	decompensated *vs* compensated cirrhosis	Serum	[25]
lipids	-	HCC *vs* adjacent tissue	Liver tissue	[39]
lipids	-	high grade HCC *vs* low grade HCC	Liver tissue	[39]
OH-butyrate	-	Cirrhosis severity	Serum	[20]
P-choline	+	Fibrosis *vs* cirrhosis	Liver tissue	[21]
P-choline	-	Mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
P-choline	+	HCC *vs* adjacent tissue	Liver tissue	[39]
P-choline	+	Cirrhosis and encephalopathy	Serum	[26]
P-choline/GPC	-	High grade HCC *vs* low grade HCC	Liver tissue	[39]
Pdt-choline	+	Cholangicarcinoma *vs* non cancer	Bile	[38]
Pdt-choline	-	Mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
P-ethanolamine	+	HCC *vs* adjacent tissue	Liver tissue	[39]
P-ethanolamine	+	High grade HCC *vs* low grade HCC	Liver tissue	[39]
P-ethanolamine	+	Fibrosis *vs* cirrhosis	Liver tissue	[21]
PUFA	-	Mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
Pyruvate	+	AIH *vs* healthy, PBC, OS and DILI	Plasma	[17]
Saturation index	+	Mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
Total lipids	+, -	Mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
Total choline/lipids	-	Mild *vs* moderate fibrosis *vs* cirrhosis	Liver tissue	[40]
Unsaturated FA	+	Fibrose *vs* cirrhose	Liver tissue	[21]
Unsaturated FA	+	Cirrhosis (HBV/Alcohol) *vs* controls	Serum	[24]

VLDL	-	Cirrhosis (HBV/Alcohol) *vs* controls	Serum	[24]
VLDL	-	Decompensated *vs* compensated cirrhosis	Serum	[25]

HBV: Hepatitis B virus; HBE: Hepatitis E virus; HBC: Hepatitis C virus; HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; OS: Overleap syndrome; PBC: Primary biliary cirrhosis; DILI: Drug induced liver injury; FA: Fatty acid; HDL: High density lipoproteins; LDL: Low density lipoproteins; VLDL: Very low density lipoproteins; P-choline: Phospho-choline; GPC: Glycerol-phospho-choline; P-ethanolamine: Phosphor-ethanolamine; PUFA: Polyunsaturated fatty acids; Pdt-choline: Phosphatidylcholine.

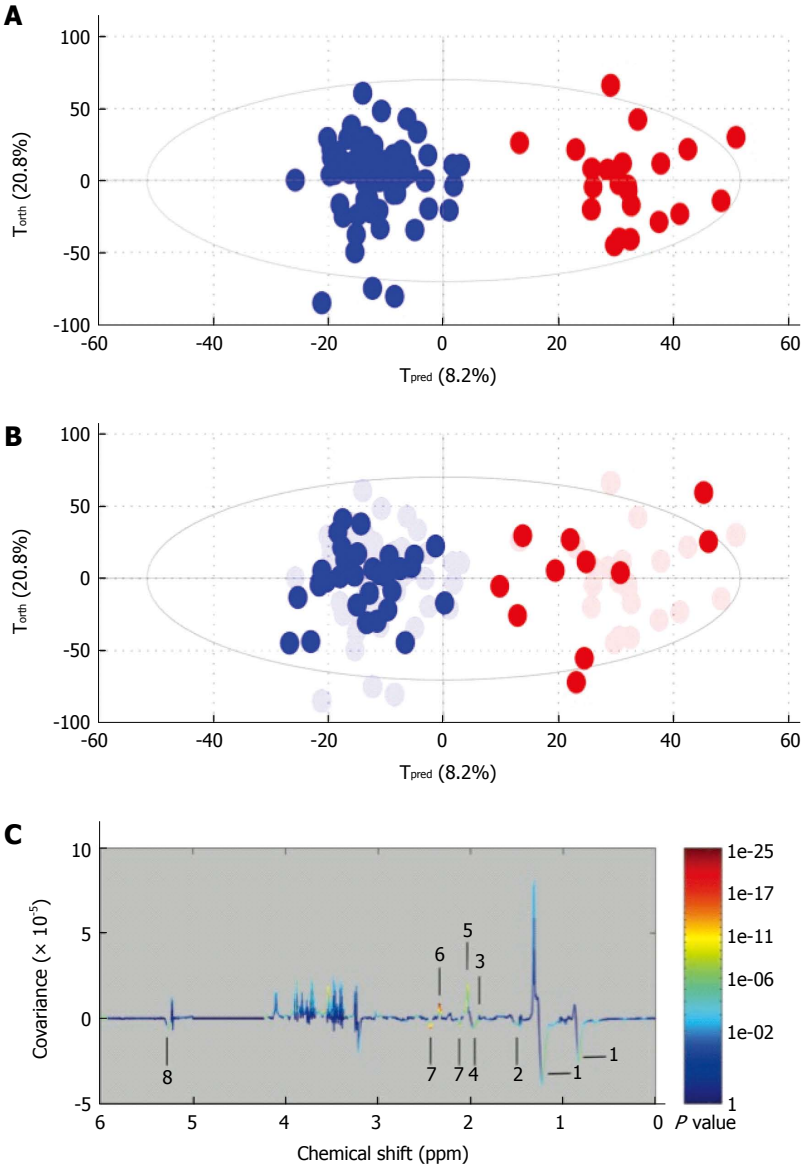

Figure 4 Example of metabolomic study using a training and test set to validate the model. A: Creation of the model. On this Figure called score plot, each point represented the projection of an NMR spectrum (and thus one patient is sample) on both axes of the model. On this score plot, each dot corresponds to a spectrum colored according to the absence (blue) or the presence (red) of hepatocellular carcinoma (HCC). The constructed model provides a good distinction between the spectrum of cirrhotic patients without HCC and those with HCC; B: Validation of the model. Each new spectrum was projected in the score plot using the previously constructed model to enable prediction of the presence or absence of hepatocellular carcinoma (HCC). Each dot corresponds to a spectrum coloured depending on the absence (blue) or presence (red) of HCC; C: Discriminant metabolites. On this Figure called loading plot, variations of bucket intensities are represented using a line plot between 0 to 6 ppm. Positive signals correspond to the metabolites present at increased concentrations in patients with large HCC. Conversely, negative signals correspond to the metabolites present at increased concentrations in patients without HCC. 1: HDL; 2: Fatty acids; 3: Acetate; 4: Fatty acids; 5: N-acetyl-glycoprotein; 6: Glutamate; 7: Glutamine; 8: Fatty acids. Adapted from Nahon *et al*[29].

with ^{1}H-NMR[27]. The hypothesis in this study was that cirrhotic patients with acute event (acute-on-chronic liver failure or ACLF group) have had a specific metabolic response as compared to cirrhotic patients with stable cirrhosis (chronic liver failure or CLF group). Both groups were distinguished using multivariable statistical methods and specific metabolomics fingerprint of ACLF patients in intensive care unit was identified. Several metabolites were identified and reflected major change in liver function such as energy metabolism, urea metabolism or amino-acid metabolism but also major extra-liver function change such as renal impairment or related to inflammation and/or necrosis.

Liver cancer: Recent studies have attempted to describe the metabolic phenotype of liver cancer in heterogeneous populations of patients with hepatocellular carcinoma (HCC) using ^{1}H NMR. This has led to the identification of various metabolically impaired pathways in serum and urine[28-31]. Goa *et al*[28] have shown that metabolite profiles obtained from ^{1}H NMR-based metabolomics analysis of blood serum may be different in healthy volunteers compared to patients with liver cirrhosis or hepatocellular carcinoma. Major changes to metabolites within the sera include lipids, ketone bodies, and amino-acid metabolism. Soper *et al*[32] have performed an *in vitro* ^{1}H NMR spectroscopy study to characterize liver biopsy samples into normal, cirrhotic or hepatocellular carcinoma on the basis of a computer-based statistical classification strategy with changes in lipids, choline and creatine identified. Ninety-eight percent of hepatocellular carcinomas in this series were distinguished from non-malignant tissue on the basis of reduced lipid and increased choline content. We used this approach to assesse the metabolomic profiles of serum from alcoholic cirrhotic patients with and without hepatocellular carcinoma[29]. This study included 154 consecutive patients with compensated biopsy-proven cirrhosis. Among these, 93 had cirrhosis without HCC, 28 had biopsy-proven HCC eligible for curative treatment ("small" HCC) and 33 had HCC outside the curative treatment criteria ("large" HCC). The first step in this study was to create a diagnostic model with "large" HCC population and validate it (Figure 4A and B). In this model, discriminant metabolites that increased with large HCC were, glutamate, acetate and N-acetyl glycoproteins, whereas metabolites that correlated with cirrhosis were lipids and glutamine (Figure 4C). The second step was to assess the diagnostic performance of the model using the "small HCC" population. Unfortunately projection of small HCC samples into the first model showed a heterogeneous distribution between large HCC and cirrhotic samples. Nevertheless, small HCC patients with metabolomic profiles similar to those of large HCC group had higher incidences of recurrence or death during follow-up (63% *vs* 47%). Serum NMR-based metabolomics identified metabolic fingerprints that could be specific to large HCC in cirrhotic livers. From a metabolomic standpoint, some patients with small HCC, who are eligible for curative treatments, seem to behave as patients with advanced cancerous disease. This finding indicates the usefulness of the monitoring by this approach during the follow-up of those patients before and after treatment.

Biliary tract cancer is an uncommon type of liver cancer with high mortality and which is difficult to diagnosis. Wen *et al*[33] used metabolomics profile of bile to distinguish patients with biliary cancer from patients with benign biliary duct diseases. This approach provides a good performance to discriminate cancer from benign diseases. Moreover, metabolomics approach has shown higher diagnostic performance (sensitivity of 88% and specificity of 81%) than conventional tests (CA19-9; CEA and bile cytology).

Liver transplantation

Clinical course after liver transplantation is sometimes chaotic and life threatening Unfortunately, bad outcomes are unpredictable, especially immediately after the transplant. One of the major concerns for the transplant team immediately after the surgery is to know whether the graft works. Failure of graft function remains an important cause of mortality. Some serial reports using metabolomics approaches have shown interesting results concerning the follow-up of patients. Serkova *et al*[34] described the blood metabolomics profile, which permits early detection of graft failure. In this case report, they were able to identify several metabolites in case of graft failure using sequential approach with multiple samples in the same patient: lactate, uric acid, glutamine, methionine increase and total fatty acids, citrate decrease. Thus, they have shown that metabolomics profiling can be a additional tool in clinical decision making. Unfortunately, validation on this interesting work is not available.

To assess the quality of the liver before transplantation, Melendez *et al*[35] analyzed metabolomic profiles of hepatic bile *in vitro* ^{1}H NMR spectroscopy. They included bile samples from donors and recipients. The main result showed a greater phosphatidylcholine signal in bile from steatotic graft compared with normal grafts.

CURRENT LIMITATION OF METABOLOMICS AND FUTURE CLINICAL APPLICATION

In this review we have highlighted studies that have advanced our understanding of various liver diseases. The examples above demonstrate that the integration of metabolomics approaches into basic and biomedical research is already improving our understanding of biological mechanism, with important applications

in the study of disease and/or therapeutic response. Technological advances have brought metabolomics to the point where these techniques can find general application in medicine.

However, there are several challenges to be overcome before metabolomics approaches can become a valuable clinical tool. It will be necessary to translate the technic in hospital lab, improve, simplify and automatized bioinformatics strategies, automatic recon of biomarker or metabolomics profile, validated the results in large prospective observational and interventional studies with meaningful clinical-end point.

Only after that and probably in a near future, results from metabolomics approach (with analysis and interpretation) will be available to the clinician in less than 1 d.

Metabolomics profiling could be useful in personalized medicine for diagnosis, prognosis and to follow patients-response before and after treatment. Metabolomics has the potential of providing new criteria to risk-stratify patients and develop novel approaches for individualized treatment.

CONCLUSION

Metabolomics is a powerful method that can be used to quantitatively assess the differences in metabolite abundance or to determine metabolic fingerprint discriminating different disease states, the severity of disease extension, drug treatment metabolic impairment or pathophysiological mechanism investigation. The ability to perform such studies in a large range of biological samples, especially urine samples, which are easy to collect and non-invasive, makes it an attractive platform for translation to clinical use.

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MINIREVIEWS

Solid, non-skin, post-liver transplant tumors: Key role of lifestyle and immunosuppression management

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Abstract

Liver transplantation has been the treatment of choice for end-stage liver disease since 1983. Cancer has emerged as a major long-term cause of death for liver transplant recipients. Many retrospective studies that have explored standardized incidence ratio have reported increased rates of solid organ cancers post-liver transplantation; some have also studied risk factors. Liver transplantation results in a two to five-fold mean increase in the rate of solid organ cancers. Risk of head and neck, lung, esophageal, cervical cancers and Kaposi's sarcoma is high, but risk of colorectal cancer is not clearly demonstrated. There appears to be no excess risk of developing breast or prostate cancer. Environmental risk factors such as viral infection and tobacco consumption, and personal risk factors such as obesity play a key role, but recent data also implicate the role of calcineurin inhibitors, whose cumulative and dose-dependent effects on cell metabolism might play a direct role in oncogenesis. In this paper, we review the results of studies assessing the incidence of non-skin solid tumors in order to understand the mechanisms underlying solid cancers in post-liver transplant patients and, ultimately, discuss how to prevent these cancers. Immunosuppressive protocol changes, including a calcineurin inhibitor-free regimen, combined with dietary guidelines and smoking cessation, are theoretically the best preventive measures.

Key words: Liver transplantation; Tumors; Calcineurin inhibitors; Immunosuppression; Risk factors; Tacrolimus; Review; Incidence

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Core tip: Liver transplantation results in two to five-fold mean increase in the rate of solid organ cancers. In this paper, we review the results of studies assessing the incidence of non-skin solid tumors in post-liver transplant patients to understand the mechanisms underlying solid cancers in these patients, and discuss how to prevent these cancers. Risk of smoking and viral-related malignancies is high, but recent data

also implicate the role of calcineurin inhibitors, whose cumulative and dose-dependent effects on cell metabolism might play a direct role in oncogenesis.

Carenco C, Faure S, Ursic-Bedoya J, Herrero A, Pageaux GP. Solid, non-skin, post-liver transplant tumors: Key role of lifestyle and immunosuppression management. *World J Gastroenterol* 2016; 22(1): 427-434 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i1/427.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i1.427

INTRODUCTION

Liver transplantation (LT) has been the treatment of choice for end-stage liver disease since 1983[1], and more than 5500 of these procedures are performed in Europe each year[2]. Infections and surgical complications are the primary causes of mortality during the early post-transplantation period. However, cancer has emerged as a major long-term cause of death in liver transplant recipients[2-5].

The rate of post-transplant lymphoproliferative disorders (PTLD) and skin cancers is 10 to 30-fold higher than for the general population[6-9]. The calcineurin inhibitors (CNIs), tacrolimus (TL) and cyclosporine, which are the cornerstones of all immunosuppressive treatments used following LT, are well-established risk factors for PTLD[10] and cutaneous cancers[11].

Recently, many studies have compared the incidence of *de novo* solid tumors in the general population to liver transplant recipients, and the risk factors involved. These studies have demonstrated that patients who receive a liver transplant often have well-established or suspected risk factors for solid cancers: (1) Tobacco and/or alcohol consumption before transplantation is extremely common in patients, especially those who undergo LT for alcoholic cirrhosis, which account for 36% of the LTs performed in Europe[2]. Continued smoking after LT is common, and resumption of alcohol consumption is not unusual; (2) CNI exposure occurs with all liver transplant patients. It promotes infection by viruses that have oncogenic potential such as human papilloma virus (HPV) and herpes human virus 8 (HHV8). CNIs may also have direct oncogenic effects; and (3) Metabolic syndromes, particularly obesity and diabetes, are common before the LT, and they are further exacerbated by exposure to CNIs after the LT.

In this review we specifically study the literature on the incidence and risk factors for non-skin solid cancers after LT.

LT RESULTS IN A TWO TO FIVE-FOLD MEAN INCREASE IN THE RATE OF SOLID ORGAN CANCERS

In an observational study using the United Kingdom transplant database[9], which contains 6771 liver transplant recipients, the standardized incidence ratio (SIR) was 2.2 for non-skin solid tumors following an LT. Similar results were found in smaller cohorts[6,8,11-17] in Italy (SIR = 2.6), the Netherlands (SIR = 4.4), Spain (SIR = 2.3), France (SIR = 3.7), and Canada (SIR = 2.5). These results are summarized in Table 1. In another Italian study[18], the incidence rate of non-skin solid tumors did not increase after LT; however, this study had the shortest median follow-up time.

Overall, the risk of developing a post-LT non-skin solid tumor is high, as confirmed by several studies comparing liver transplant recipients with the sex and age-matched general population, both in large-scale registry studies and single-center studies. Median time to develop post-LT non-skin solid tumor was 4.2 years in Baccarani[8] cohort and 5 years in Haagsma[4] and Aberg[6] cohorts. In our cohort of 465 patients[13], the median time to diagnosis of solid cancers after LT was 6.3 ± 4.3 years (6 years median). Indeed, sufficient follow-up time is necessary in order to highlight the elevated risk of solid tumors.

INCIDENCE AND RISK OF SITE-SPECIFIC CANCERS

Risk of smoking-related malignancies is high

Head and neck cancers have the highest increased risk in all of the European cohorts (SIR of 2.7-15.8, Table 1). A non-significant increased risk was also found in Canadian[14], Taiwanese[15], and Japanese[16] studies.

An elevated risk of lung cancer has been established, although it is not encountered in all studies. Indeed, in the United States the NKKD cohort[7] demonstrated a SIR of 1.9 for lung cancer in 37958 liver transplant recipients, and a SIR of 1.6 was found in the United Kingdom transplant registry of 6771 patients[9].

Three studies[8,13,16] found an increased risk of esophageal cancer (SIR of 10.5-23.4).

Finally, the risk of cancer of the urological tract was significantly higher in three studies[12,15,17] after LT (SIR of 2.9-6.2).

Risk of virus-related malignancies is well established

The relative risk of developing Kaposi's sarcoma was high in two Italian studies, with a SIR of 144 and 128 for the Baccarani *et al*[8] and Maggi *et al*[17] studies, respectively; however, this cancer is an exception and it mainly occurs in Mediterranean populations. It is related to HHV8 infection. The highest incidence is found in Saudi Arabia, and the lowest incidence is in Western nations, such as the United States[19].

In a study by Baccarani *et al*[8] an excessive risk of cervical malignancies was found, with a SIR of 30.7; this was probably related to HPV infection.

Risk of colorectal cancer is probably increased

The excess risk of colorectal cancer (CRC) remains

Table 1 Standardized incidence ratio (95%CI) of non-skin solid cancer after liver transplantation, *n* (range)

Ref.	LT patients (*n*)	Country	Median follow-up time (yr)	All non-skin solid tumors	Head and neck	Lung	Colorectal	Anal	Esophageal	Pancreatic	Kidney	Prostate	Urological tract	Kaposi's sarcoma	Breast	Cervical
Engels *et al*[7]	37958	United States				1.9 (1.7-2.2)					1.8 (1.4-2.3)					
Collett *et al*[9]	6771	United Kingdom		2.2 (2-2.4)	10 (5.9-16)	1.6 (1.2-2.2)	2.3 (1.7-3)	3.3 (0.4-12)			1.8 (0.8-3.6)			0	0.8 (0.5-1.1)	
Ettorre *et al*[18]	1675	Italy	5.2	0.9 (0.7-1.2)	4.5 (2.7-7.1)	1.1 (0.9-1.9)	1.2 (0.6-2.2)			1.1 (0.3-3.9)			0.8 (0.3-1.6)		0.7 (0.1-1.9)	
Aberg *et al*[6]	540	Finland	6.3		14.8 (0.4-82)	0	1.6 (0.2-5.7)			2.3 (0.1-13)	4.2 (0.5-15)	1.2 (0.2-4.5)			0.3 (0.1-1.4)	
Herrero *et al*[12]	297	Spain	6.5	2.34 (1.7-3.2)	4.1 (1.7-8.5)	2.4 (0.6-4)	2.5 (1.3-3.5)						2.9 (1.7-4.8)			
Baccarani *et al*[8]	417	Italy	6.8	2.6 (1.9-3.6)	7 (3-13.7)	1.6 (0.4-4.1)	1.4 (0.2-5.1)		23.4 (4.6-55)					144 (53-313)	0.6 (0-3.4)	30.7 (6.3-90)
Jiang *et al*[14]	2034	Canada		2.5 (2.1-3)	2.5 (0.5-7.3)	1.4 (0.7-2.6)	2.6 (1.4-4.4)			3.3 (0.7-9.6)	3.1 (0.8-7.9)	1 (0.3-2.4)			0.6 (0.2-1.4)	
Haagsma *et al*[4]	174	The Netherlands	5.1	4.4 (2.4-7.3)	2.7 (1.2-5.2)		12.5 (2.5-36)				30 (6.1-87)					
Hsiao *et al*[15]	444	Taiwan	4.2		0 (0-5.7)	1.9 (0.2-6.7)	0 (0-2.84)			6.2 (0.1-34)			10.2 (1.1-36)	0	2.3 (0.3-8.4)	
Kaneko *et al*[16]	360	Japan	7.5		3.7 (0.5-26)		3.5 (1.8-7)		16.9 (2.4-18)	6.4 (1.6-25)		2.2 (0.6-8.9)			0.9 (0.1-6.4)	
Maggi *et al*[17]	494	Italy	7.2	2 (1.4-2.9)	3.4 (0.9-8.8)	2.1 (0.8-4.6)	1.6 (0.3-4.6)					1.6 (0.4-4.1)	2.9 (1-6.9)	128 (51-263)	1 (0.2-2.9)	5.7 (0.1-32)
Carenco *et al*[13]	465	France	7.8	3.7 (2.8-4.9)	15.8 (9.4-27)	5.1 (2.9-9)	2.7 (1.3-5.6)		10.5 (3.9-28)					0		

SIR: Standardized incidence ratio.

unclear. An increased risk was found in four European studies[4,9,12,13], one Japanese study[17], and one Canadian study[14]. This risk was not observed in three Italian studies[8,17,18] and one Taiwanese study[15]. However, in a meta-analysis, Sint Nicolaas[20] found that the risk of developing CRC was 2.5 times higher (95%CI: 1.65-4.05) in liver transplant recipients.

Excess risk of developing kidney, pancreatic, or brain cancer is not proven

The risk of developing kidney cancer after LT remains unclear. In the NKKD cohort[7] and a cohort in a study by Haagsma *et al*[4], the incidences of kidney cancer were significantly higher than expected compared with the general population. However, this was not the case for a cohort in the United Kingdom[9], and in two other studies[6,13].

Only one Japanese study[16] has found a significantly increased risk of pancreatic cancer after LT, but this result has not been confirmed by other studies.

To the best of our knowledge, there are no solid data regarding the development of brain cancer in liver transplant recipients. Engels[7] did not show excess risk in 175732 organ transplant recipients.

There is no excess risk of developing breast or prostate cancer

No study to date has shown an increased risk of prostate or breast cancer after LT, although these are among the most common malignancies in the general adult population. Indeed, in a meta-analysis of 31977 solid organ transplant recipients (97% were renal transplants)[21] there was no evidence for a significantly increased SIR for breast or prostate cancer.

Prospective cohort studies with large numbers of liver transplant recipients, a rigorous collection of *de novo* solid cancers after LT, risk factor data, and sufficient follow-up times are necessary to obtain accurate information about the risk of each site-specific cancer. Indeed, current data do not allow elucidation of the risk of kidney, brain, stomach, pancreatic, and anal cancer after LT.

Table 2 Risk factors for non-skin solid tumors after liver transplantation from multivariate analyses

	Ref.	Risk factor	Associated cancer	SIR, HR, RR, or OR (95%CI)
Viral infection	Baccarani *et al*[8]	HPV exposure	Cervical	SIR = 30.7 (6.3-90)
	Collett *et al*[9]		Anal	SIR = 3.3 (0.4-12)
	Baccarani *et al*[8]	HHV8 exposure	Kaposi's sarcoma	SIR = 144 (53-313)
	Maggi *et al*[17]			SIR = 128 (51-263)
Demographic data	Herrero *et al*[22]	Recipient's age	All non-skin tumors	HR = 1.90 (1.32-2.73)
	Watt *et al*[23]		All non-skin solid tumors	HR = 1.33 (1.05-1.66)
	Herrero *et al*[34]		Smoking-related tumors	HR = 1.09 (1.03-1.15)
Indication for LT	Watt *et al*[23]	Alcohol cirrhosis	All non-skin solid tumors	HR = 2.14 (1.22-3.73)
		Primary sclerosis cholangitis	All non-skin solid tumors	HR = 2.62 (1.50-4.56)
Lifestyle	Benlloch *et al*[53]	Alcohol consumption	All non-skin tumors	RR = 3 (1.5-5.8)
	Herrero *et al*[22]			HR = 2.87 (1.15-7.19)
	Herrero *et al*[22]	Tobacco consumption	All non-skin tumors	HR = 3.07 (1.32-7.16)
	Watt *et al*[23]		All non-skin solid tumors	HR = 1.72 (1.06-2.79)
	Carenco *et al*[13]			OR = 5.5 (2.5-12)
	Herrero *et al*[34]		Smoking-related tumors	HR = 19.17 (4.17-88.10)
	Carenco *et al*[13]			OR = 14.7 (1.8-119)
	Carenco *et al*[13]	Obesity	All non-skin solid tumors	OR = 2.2 (1.1-4.3)
Immunosuppression	Carenco *et al*[49]	Mean tacrolimus TC during first year post-LT	All non-skin solid tumors	OR = 2.01 (1.57-2.59)

SIR: Standardized incidence ratio; HPV: Human papilloma virus; HHV8: Herpes human virus 8; HR: Hazard ratio; RR: Relative risk; OR: Odds ratio; CI: Confidence interval; TC: Through concentration.

RISK FACTORS FOR NON-SKIN SOLID MALIGNANCIES

Environmental risk factors

Viral infection: In a meta-analysis involving 31977 solid organ transplant recipients (97% of whom were renal transplants) Grulich[21] demonstrated a high risk of HHV8-related cancer (Kaposi's sarcoma) and HPV-related cancer (cervical, anal, vulval, vaginal, and penile cancer, as well as head and neck cancer) in these immunocompromised patients. In this study, similar results were found for people with HIV/AIDS. This further supports the notion that the risk of infection with an oncovirus and, consequently, the risk of cancer, is increased in immunocompromised patients (Table 2).

Is this also the case for liver transplant recipients who require a lower level of immunosuppression than that received by kidney transplant recipients? Kaposi's sarcoma is rare in the general population; several studies have described an incidence of 0.5%-2.8% for this disease after LT[22-24]. As shown by the Italian studies[8,17], Kaposi's sarcoma occurs much more frequently in patients living in areas where HHV8 is endemic[25,26], compared to the general population, while none of the 6846 liver transplant recipients developed this cancer in the United Kingdom cohort[9].

Out of 417 post-LT patients, Baccarani[8] encountered three patients with cervical cancer (0.7%), which was 30 times more than expected. It has been shown that before solid organ transplant, 29% of patients were infected with a high-oncogenic potential HPV serotype[27]. Moreover, it is now established that HPV infection is a risk factor for epidermoid head and neck carcinomas[28], which could partly explain the high rate of these cancers after LT.

Long-term immunodeficiency places liver transplant recipients at risk of oncoviral infection, which is conducive to malignancy and necessitates efficient management of the immunosuppressive therapy.

Alcohol and tobacco consumption: For the general population, tobacco and alcohol consumption are known risk factors for oral, pharyngeal, laryngeal, esophageal, and upper airway tumors[29-32]. There is a synergistic effect when patients are exposed to both tobacco and alcohol; the risk of these tumors is more than seven times higher in heavy drinkers and smokers[30-33].

Using a multivariate analysis in a retrospective study of 722 liver transplant patients, previous alcohol abuse was associated with a three-fold risk of developing a *de novo* tumor following LT (P = 0.002, 95%CI: 1.5-5.8)[33]. In a smaller cohort, using a multivariate model, Herrero *et al*[22] found a hazard ratio of 2.87 (95%CI: 1.15-7.19) of developing a non-skin tumor after LT among patients who consumed large amounts of alcohol. In two other studies[17,23], patients who received a transplant for alcoholic cirrhosis had a higher risk of non-skin solid cancers after LT, but alcohol consumption was not an independent cancer risk factor, unlike tobacco use. We found similar results in a study with 465 patients[13]: using a univariate analysis, alcohol consumption was a risk factor for developing a solid cancer, but in multivariate analysis it was not an independent risk factor, unlike tobacco consumption and obesity before LT.

A history of smoking is common in patients who undergo LT for alcoholic liver disease, and tobacco consumption is now an independent risk factor for the development of a non-skin solid cancer after LT[13,22,23]. Herrero *et al*[34] specifically described the incidence and risk factors for "smoking-related malignancies" (SRM), defined as head and neck, esophageal, kidney, and urinary tract carcinomas, in 339 liver transplant recipients. Compared to a sex and age-matched general population, they observed a relative risk of 8.5 for the development of SRM in active smokers, and 4.4 in former smokers *vs* 0.36 in patients who never smoked. In a multivariate analysis, significant smoking was an independent risk factor, with a hazard ratio of 19.

Interestingly, in our cohort of 465 liver transplant recipients[13], 38 patients developed an SRM, and tobacco consumption before and after the LT were the only independent risk factors found when using a multivariate analysis. Therefore, it is paramount that all patients cease tobacco and alcohol consumption prior to and after LT.

Personal risk factors

Age and gender: Age is a well-established risk factor for solid cancer in the general population. Using a multivariate analysis, Herrero *et al*[22] and Watt *et al*[23] concluded that this is also the case for liver transplant recipients. In our series of 465 patients[13], we did not find this to be a risk factor, probably due to the low standard deviation of age within our patient cohort.

Numerous single-center studies have failed to find a statistically significant difference between male and female liver transplant recipients in terms of the development of *de novo* solid tumors using a multivariate analysis after LT[13,15,22,23]. This is probably because of the much greater weight of other risk factors.

Obesity: Liver transplant recipients often present with a metabolic syndrome before transplantation, or develop it after the procedure; these syndromes can be triggered and aggravated by anti-calcineurins and corticosteroids[35].

In our series of 465 patients[13], 27.4% of the 65 patients who developed a non-skin solid tumor after LT were obese, *vs* 15.8% of the rest of the cohort. Using a multivariate analysis, we found that obesity and tobacco consumption before LT were independent risk factors for non-skin solid tumors. Interestingly, in a subgroup analysis of 427 patients with 27 different cancers (eight colorectal, eight prostate, four breast, and seven other types of cancer), obesity was the only independent risk factor after excluding smoking-induced cancer (head and neck, lung, esophageal, and urinary tract cancer). To the best of our knowledge, there are no other studies that have investigated this risk factor after LT, although obesity and excess body weight are independent risk factors for breast, endometrial, esophageal, and colorectal cancers in the general population[36].

In addition to cardiovascular complications that can cause obesity, it seems that obesity could be responsible for non-skin solid cancers after LT. Regular physical activity and a balanced diet are essential for these patients.

Specific LT risk factors

Indications for LT: The incidence of CRC after LT differs depending on the series; it has been found to range from 0.03% to 3.1%[4-9,16,23,37]. This spread can be explained by the proportion of patients who received a transplant for primary sclerosing cholangitis (PSC) in association with chronic inflammatory bowel disease (IBD). Indeed, Watt *et al*[23] found 25 cases of CRC in 798 liver transplant recipients (3.1%); 127 (15.9%) of these patients received LT for PSC. This variable was the strongest risk factor for developing a solid cancer after LT, and reflects the high risk of CRC in patients with IBD.

In Europe, PSC represents 4% of LT indications[2], yet in a study of a large series the incidence of CRC was two to three times higher than that observed in the general population[9]. This was also observed in a single-center study in Japan[16], with one patient transplanted for PSC. In our series of 465 patients[13], only six patients received a transplant for PSC, and none of the patients developed CRC; however, in the entire study, we found an incidence of CRC that was 2.7 times higher than expected. Moreover, in a meta-analysis excluding patients transplanted for PSC, Sint Nicolaas[20] found a 1.8-fold higher risk of CRC after LT.

Why do non-PSC liver transplant recipients appear to have an increased risk of CRC? John Cunningham virus (JCV) reactivation in adenomas could be a possible mechanism for this increased risk[38]. Another possibility could be the presence of precursor lesions for CRC before the LT. A case-control study found that 7.3% of the 82 liver transplant recipients developed advanced neoplasia, compared to 1.2% in the 82 control patients from the general population[39]. Another study retrospectively identified 92 liver transplant recipients who underwent a screening colonoscopy; the relative risk for advanced neoplasia was 8.9 compared to a large asymptomatic cohort[40].

CNI exposure: As well as their ability to promote infection by viruses with oncogenic potential, there is evidence from animal studies that suggests CNIs also have carcinogenic potential. This may be caused by activation of the Ras pathway[41], induction of tumor growth and metastatic potential from TGF-β1 activation[42,43], and disruption of angiogenesis and

apoptosis[44-46].

In a study of kidney transplant recipients by Dantal *et al*[47] the incidence of cancer (mainly skin malignancies) was higher in patients with elevated cyclosporine target levels (*e.g.*, 150-250 ng/mL *vs* 75-125 ng/mL). More recently, in liver transplant recipients, Vivarelli *et al*[48] found a higher risk of hepatocellular carcinoma recurrence in patients exposed to higher doses of anti-calcineurin, either cyclosporine or TC, during the first three months after LT.

We monitored the blood concentrations of TC in 247 patients treated with TC for at least one year after LT[49]. The mean TC concentration during the first year after LT was significantly higher in patients who developed non-skin solid tumors (10.3 and 7.9 ng/mL, respectively, $P < 0.0001$). The independent risk factors for developing solid cancer using a multivariate analysis were tobacco consumption before the LT, and the mean annual TC concentration during the first year after LT. Indeed, a model that takes into account smoking and mean TC concentration during the first year after LT strongly predicted the occurrence of a solid cancer in our sample population.

How can we change our immunosuppression strategies to prevent cancer? In liver transplant recipients, short-term complications are dominated by infections and post-operative complications; rejection is less problematic than in other organ transplantations. Acute cellular rejection is a rare event that may be easily controlled either with an increase in TC or a bolus of steroids.

Therefore, it might be interesting to rapidly decrease CNI concentrations to minimize the risk of solid cancer after LT. To avoid organ rejection, one could use drugs that block proliferative signaling, such as mTOR inhibitors. mTOR inhibitors have major immunosuppressive activity through their intracellular binding to FKBP12 and inhibition of mTORC1, which blocks cell cycle progression and IL-2 signaling in T cells. Indeed, these are already used in patients with CNI-related nephrotoxicity. As well as their immunosuppressive properties, these drugs have anti-oncogenic effects in preclinical models, and they are currently being investigated as anti-cancer agents in clinical trials[50]. For kidney transplantations, the CONVERT trial[51] has demonstrated lower cancer rates (mainly skin cancer) in renal allograft recipients who were switched to a sirolimus-based, CNI-free immunosuppressive treatment.

However, mTOR inhibitors have significant side effects, including rejection, delayed wound healing, mouth ulcers, and leg edema, and there is a 46% discontinuation rate among renal transplant recipients[52]. Therefore, data for liver transplant recipients from large cohorts are needed to accurately determine the risk/benefit balance of using mTOR inhibitors before they can become routine treatment for all patients.

CONCLUSION

In conclusion, *de novo* malignancy is currently the second-leading cause of death for liver transplant recipients after cardiovascular complications, and the risk of developing a non-skin solid tumor is high. This risk is higher than that observed in the general population, especially for smoking-induced cancers (head and neck, lung, and esophageal), CLC, and virus-induced cancers (cervical and Kaposi's sarcoma).

While the role of alcohol and tobacco consumption in this high rate of solid cancers is indisputable, recent data also implicate the role of CNIs, whose cumulative and dose-dependent effects on cell metabolism might play a direct role in oncogenesis. Therefore, it is paramount that LT patients cease alcohol and tobacco consumption before and after transplantation, and that the minimum dose of CNI is administered to reduce the risk of malignancy, while still preventing graft rejection.

In the future, we will evaluate the safety and efficacy of CNI-free regimens through prospective studies.

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MINIREVIEWS

Endoscopic submucosal tunnel dissection for large superficial esophageal squamous cell neoplasms

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Abstract

Endoscopic submucosal dissection (ESD) is a well-established treatment for superficial esophageal squamous cell neoplasms (SESCNs) with no risk of lymphatic metastasis. However, for large SESCNs, especially when exceeding two-thirds of the esophageal circumference, conventional ESD is time-consuming and has an increased risk of adverse events. Based on the submucosal tunnel conception, endoscopic submucosal tunnel dissection (ESTD) was first introduced by us to remove large SESCNs, with excellent results. Studies from different centers also reported favorable results. Compared with conventional ESD, ESTD has a more rapid dissection speed and R0 resection rate. Currently in China, ESTD for large SESCNs is an important part of the digestive endoscopic tunnel technique, as is peroral endoscopic myotomy for achalasia and submucosal tunnel endoscopic resection for submucosal tumors of the muscularis propria. However, not all patients with SESCNs are candidates for ESTD, and postoperative esophageal strictures should also be taken into consideration, especially for lesions with a circumference greater than three-quarters. In this article, we describe our experience, review the literature of ESTD, and provide detailed information on indications, standard procedures, outcomes, and complications of ESTD.

Key words: Endoscopic submucosal tunnel dissection; Esophageal squamous cell neoplasms; Digestive endoscopic tunnel technique; Endoscopic submucosal dissection

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Core tip: The digestive endoscopic tunnel technique

(DETT) innovatively broke the traditional boundaries between medicine and surgery and has been a recent research hotspot. Based on the submucosal tunnel concept, endoscopic submucosal tunnel dissection (ESTD) was introduced by us to treat large superficial esophageal squamous cell neoplasms, with excellent results. Studies from different centers also achieved favorable results, and ESTD has become an important part of DETT in China. Therefore, we conducted a literature review and provided detailed information on indications, standard procedures, outcomes, and complications of ESTD.

Zhai YQ, Li HK, Linghu EQ. Endoscopic submucosal tunnel dissection for large superficial esophageal squamous cell neoplasms. *World J Gastroenterol* 2016; 22(1): 435-445 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i1/435.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i1.435

INTRODUCTION

Endoscopic submucosal dissection (ESD) is acknowledged as the standard treatment for superficial esophageal squamous cell neoplasms (SESCNs)[1-3]. Compared with conventional endoscopic mucosal resection (EMR), ESD enables *en bloc* resection and precise pathological assessment, leading to a lower local recurrence rate[4]. However, with respect to large SESCNs, some frustrating problems arise, especially for tumors with a circumference that exceeds two-thirds of the esophageal lumen. During submucosal injection, rapid diffusion of submucosal liquid cushion after circumferential incision made the lifting-effect unsatisfactory. The submucosal endoscopic view was also not clear because the resected mucosa shrank and blocked the confined lumen[5,6]. Consequently, the ESD procedure is time consuming, has a high risk of adverse events, and requires highly skilled endoscopists. To overcome these problems, some endoscopic innovations were introduced, such as modified fishing-line traction system[6], peroral traction-assisted technique[7], clip-band technique[8], and medical ring system[9], but these were not suitable for extensive standardized application.

Early in 2009, Linghu *et al*[10,11] attempted to dissect a submucosal tunnel and successfully achieved *en bloc* removal of an 8 cm long circumferential SESCN. The results were presented as the "tunnel technique for circumferential esophageal lesions", at the 2009 Beijing Annual Meeting of Digestive Endoscopy. The innovative technique was termed endoscopic submucosal tunnel dissection (ESTD)[5]. Although derived from ESD, ESTD with the submucosal tunnel concept changed the traditional procedures for ESD: marking-injection-circumferential incision-submucosal dissection became a new treatment strategy for superficial esophageal neoplasms.

The submucosal tunnel formed a bridge between medical treatment and surgery, which was a long-held ambition of endoscopists. Peroral endoscopic myotomy (POEM) for achalasia launched a new field in endoscopy of digestive endoscopic tunnel technique (DETT)[12]. Inspired by POEM, submucosal tunnel endoscopic resection (STER) was developed for the treatment of submucosal tumors of the muscularis propria (MP)[13,14]. Since we first reported our experience in ESTD[5], an increasing number of endoscopists have focused on the new treatment strategy for SESCNs[15-20]. Some believe that standardized ESTD has made esophageal ESD straightforward and less difficult, especially for Western endoscopists[15]. Currently in China, ESTD has become an important part of DETT, together with POEM and STER[10].

In this review, we describe the indications, procedures, outcomes, complications, advantages, and future perspectives of ESTD for SESCNs.

INDICATIONS

Generally, whether endoscopic resection is preferred for patients with SESCNs is determined by risk of lymph node metastasis and technical resectability[21]. Postoperative quality of life also should be taken into consideration. According to 2012 Japan Esophageal Society (JES) guidelines for treatment of esophageal carcinoma[22], lesions limited to the mucosal epithelium (m1) or the lamina propria mucosa (m2) have a low risk of lymph node and distant metastasis, and radical resection can be achieved endoscopically, with similar long-term survival to surgery. Therefore, these lesions are considered to be an absolute indication for endoscopic resection. As the risk of lymphatic metastasis increases to 10%-15%, endoscopic resection is relatively indicated for lesions invading the muscularis mucosae (m3) or submucosal layer < 200 μm (sm1), although Western endoscopists remain cautious and conservative (Figure 1)[1].

Technical resectability is often determined by circumferential extension of lesions, which is an important risk factor for postoperative stenosis[23,24]. As a result of advances in endoscopic techniques, the 2012 JES guidelines removed the restriction of lesion circumference in the 2007 edition, by which endoscopic resection was only indicated for m1 or m2 lesions not exceeding two-thirds of the esophageal circumference (absolute indication)[22,25]. We believe that it was the standardized ESTD that enabled lesion size no longer to be a barrier to endoscopic resection and significantly improved efficacy. Double-tunnel ESTD could further minimize operation duration for whole circumferential lesions[20]. Despite that, patients with lesions larger than three-quarters of the circumference should be informed of the risk of esophageal stenosis and prophylactic measures should be taken[23,24,26]. Lesions < 15 mm in diameter are not recommended for ESTD, although the technique is reported to be feasible[15].

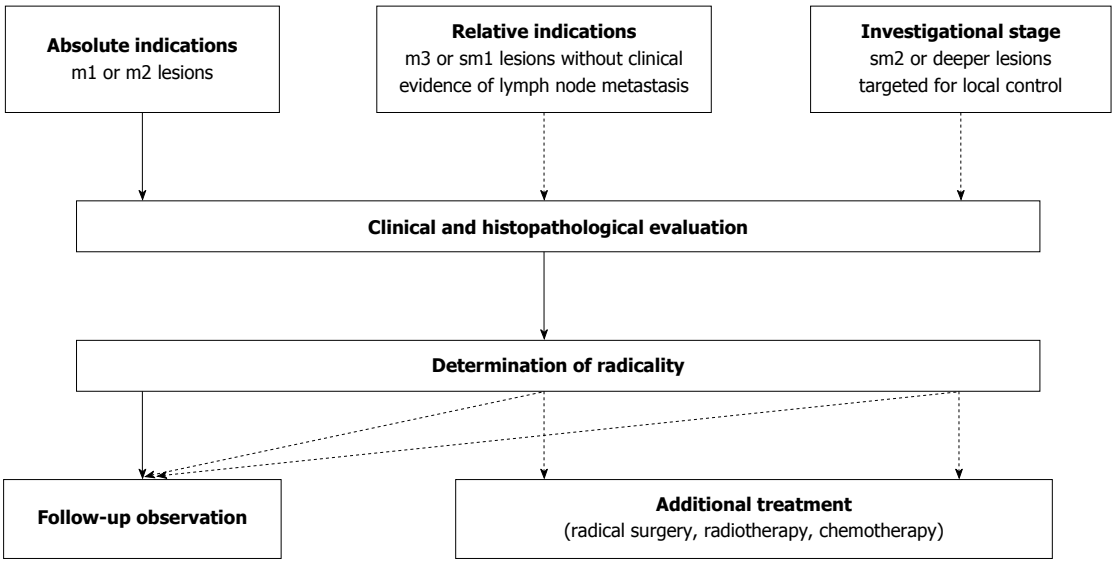

Figure 1 Indications for endoscopic resection by 2012 Japan Esophageal Society guidelines.

The reasons are as follows: (1) EMR is easy to perform and achieves a favorable *en bloc* resection rate; and (2) excess normal mucosa is resected by the creation of a submucosal tunnel and is followed by more trauma and longer recovery. Based on calculation and our experience, the diameter of the submucosal tunnel should be ⩾ 10 mm, and the resected area should be ⩾ 20 mm in diameter, or one-third of the circumference[10,27].

Therefore, ESTD is indicated for: (1) lesions not invading deeper than sm1 and without clinical evidence of lymph node metastasis; and (2) lesions at least one-third of the esophageal circumference and ⩾ 20 mm in diameter.

EQUIPMENT REQUIRED

A forward-viewing gastroscope (GIF 260J; Olympus, Tokyo, Japan) with a water-jet function is used, with a 9.8 mm outer diameter and a single 3.2 mm working channel. A transparent cap (D201-11804; Olympus) is fitted to the tip of the endoscope to provide a clear submucosal view. It also facilitates entering the tunnel and blunt dissection. Similar to ESD, an electrosurgical energy generator (ICC-200 or VIO 300D; Erbe, Tübingen, Germany) is connected to provide cutting or coagulation when needed. Various electrosurgical knives are available, including the dual knife, insulated-tip (IT) knife, the triangle-tip (TT) knife, and hook knife. The hybrid or flush knife, with a combined function of submucosal injection and electrosurgical knife, avoids frequent changing of tools, making the procedure simpler and more efficient. Selection of knives depends on the preference and specialty of the endoscopist. We prefer the dual knife for marking and mucosal incision and the IT knife for submucosal dissection and lateral resection.

A hemostatic forceps is used to handle active bleeding or large exposed vessels when a hand-held knife is insufficient. CO_2 insufflation is strongly suggested because it decreases the risk of air-related adverse events, such as subcutaneous or mediastinal emphysema and even pneumothorax, and for its rapid absorption rate, around 150 times higher than air[28-30].

ESTD PROCEDURES

Patients are placed in the left lateral position. General anesthesia with mechanical ventilation is required in view of lesion location and long operation time. Endotracheal intubation prevents aspiration, and positive pressure ventilation reduces the risk of air-related adverse events[5,31]. ESTD procedures differ from ESD in the following ways (Figures 2 and 3)[5].

Marking lesion margin

Magnifying narrow-band imaging and lugol staining are carried out to delineate the lesion. Dots are made around 5 mm outside the margin with argon plasma coagulation (APC) or electrosurgical knives. For circumferential lesions, circular dots reveal the anal and oral margins.

Submucosal injection and mucosal incision

In the anal-oral sequence, the anal side of the mucosa is first cut open transversely with a dual knife after the submucosal fluid cushion reaches an acceptable level. In China, the frequently used injection fluid is a mixture of 100 mL normal saline solution or glycerol-fructose injection, 1 mg epinephrine, and indigo carmine or methylene blue, for its ease of application and low cost. Highly viscous hyaluronic acid can maintain a thick fluid cushion for a long time and is widely used by Japanese endoscopists. Subsequently, the same procedure is performed for incision of the oral side of the mucosa along the marked dots.

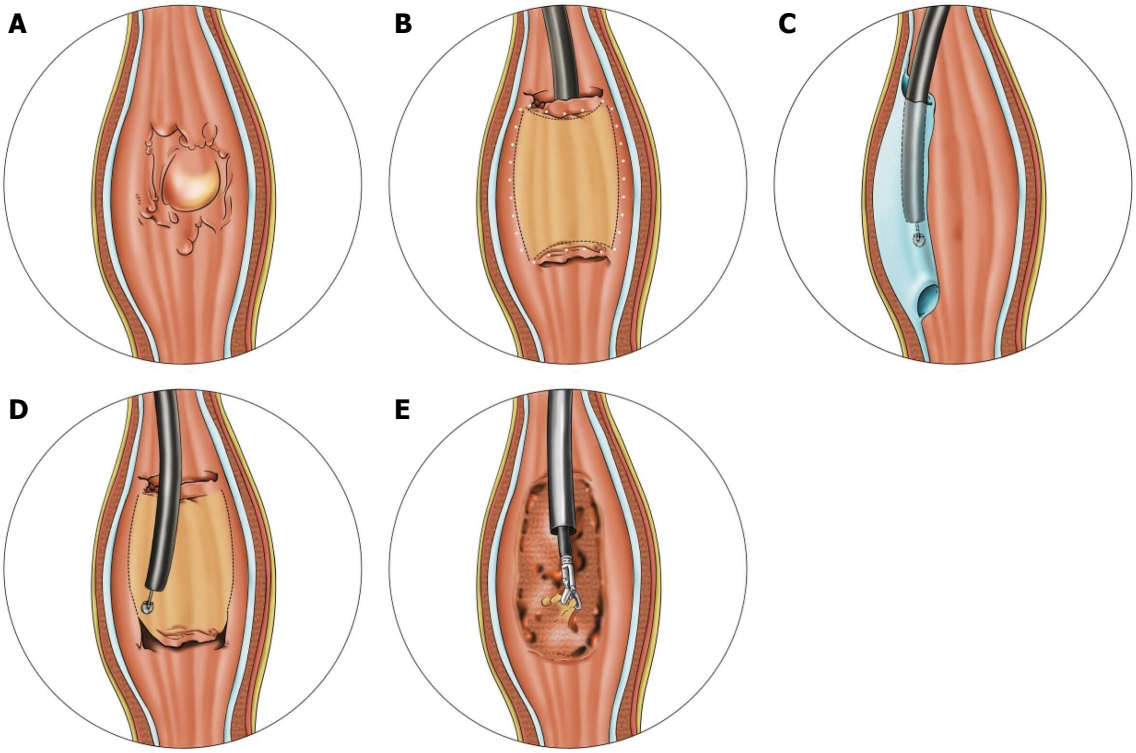

Figure 2 Schema of endoscopic submucosal tunnel dissection. A: Evaluating and delineating the neoplasm; B: After marking the lesion margin, mucosal incision was performed in the anal-oral sequence; C: A submucosal tunnel was created from the oral to anal side; D: Lateral resection with an insulated-tip knife for complete removal of the lesion; E: Preventive coagulation on artificial ulcer.

The anal side of the incision is a useful indicator of submucosal tunnel endpoint. More importantly, communication between the submucosal tunnel and esophageal lumen prevents gas accumulation and sharply rising tunnel pressure during dissection, avoids excessive normal mucosal separation, and reduces risk of air-related adverse events[5,32].

Creation of submucosal tunnel

Submucosal dissection was used to create a tunnel from the oral side to the anal side. Repeated submucosal injection is necessary to separate the mucosa from the MP to maintain an adequate space. IT knife (IT 2 or IT nano) with a small ceramic ball is a safe and efficient tool, offering both lateral and backward dissection. Submucosal dissection should be conducted close to the MP, where rich vascular networks are absent, which differs from the upper submucosal layer and muscularis mucosae. According to basic principles of DETT, at least one side of the submucosal tunnel should be intact as the only barrier to the mediastinum. Therefore, the blade should be parallel to the MP as much as possible and catch the submucosal fibers to the center of tunnel for electric cut to avoid MP injury. During lateral dissection of the tunnel, mucosal dots are a reminder of the tunnel boundary, and constant withdrawal of the endoscope from the tunnel also ensures that the tunnel is a consistent size, to avoid postoperative stenosis caused by excessive dissection.

Lateral resection

After completion of the tunnel, the endoscope is withdrawn, and the IT knife is used for lateral mucosal resection close to the markings from the anal to the oral side, until complete removal of the lesion. The resection is carried out simultaneously on the both sides of the tunnel. In this way, traction of the contralateral mucosa makes the procedure easier.

Management of artificial ulcer

After removal of the lesion, hemostatic forceps and APC are applied to coagulate the visible vessels on the surface of the artificial ulcer to prevent delayed bleeding. More attention should be given to vessels on the edge of the ulcer. Preventive clips should be placed when the MP layer is injured. Fibrin sealant or sucralfate can be used to protect the ulcer. For lesions more than three-quarters of the circumference, a fully covered esophageal metallic stent is conventionally placed to prevent postoperative stenosis in our endoscopy center.

As for double-tunnel ESTD, the difference is that circular incisions are successively performed at the anal and oral margin after marking. Then, the two opposite tunnels are dissected one after another from the oral to anal side. The procedures of double-tunnel ESTD are detailed in Figure 4.

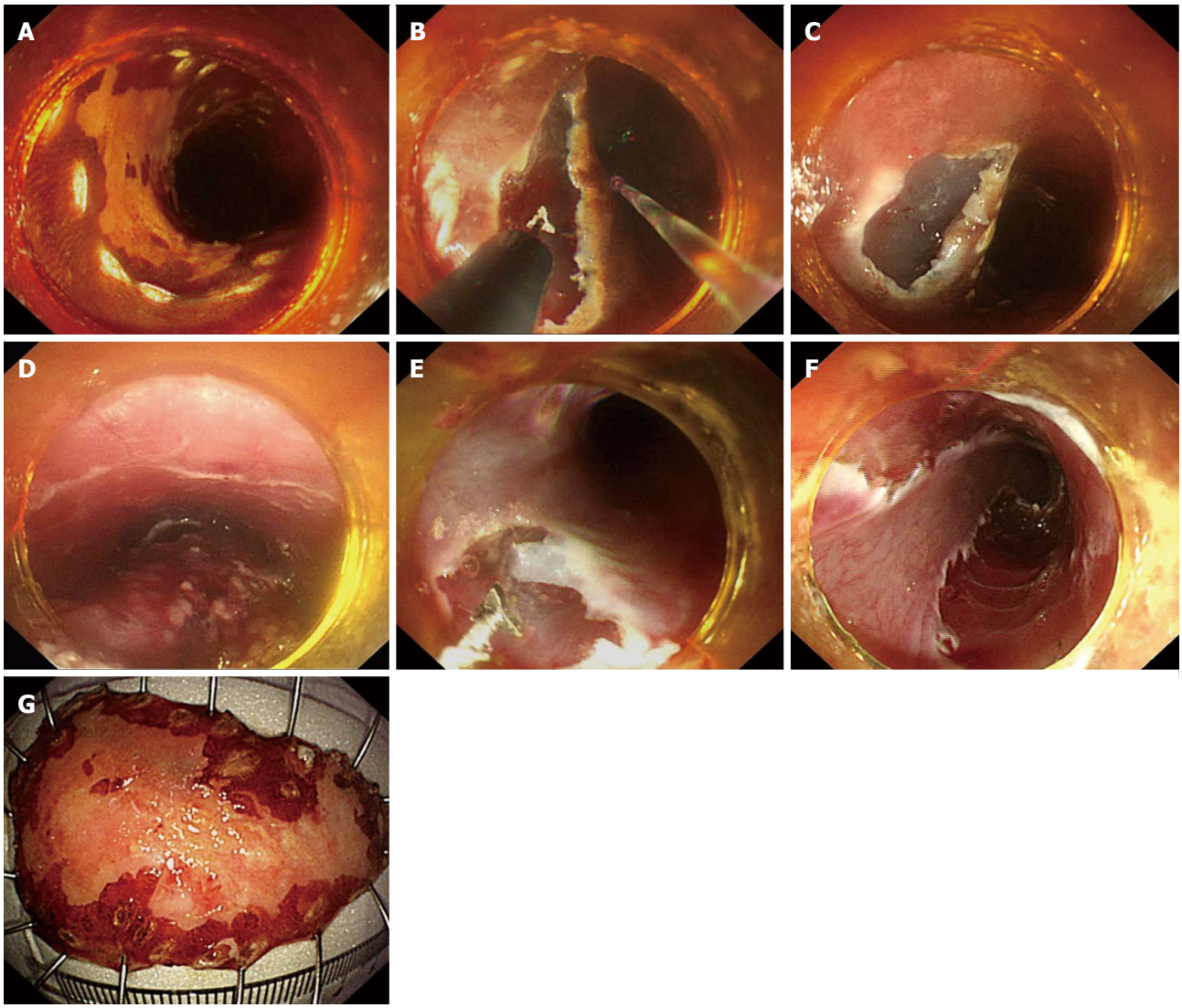

Figure 3 Endoscopic submucosal tunnel dissection with a single triangle-tip knife. A: Lugol staining to delineate the lesion, occupying almost half of the esophageal lumen. The margin was marked by argon plasma coagulation; B, C: Anal and oral incision was performed successively with a triangle-tip (TT) knife. Water jet helped entry into the submucosal tunnel; D: Submucosal tunnel was created from the oral to anal side; E: Lateral resection with a TT knife from the oral to anal side until reaching the anal incision; F: Complete *en bloc* resection was achieved in 52 min; G: The specimen, around 55 mm × 35 mm in size, was retrieved. Histopathological examination revealed a microinvasive squamous carcinoma limited in the lamina propria mucosa, free of lateral and vertical margin.

OUTCOMES AND COMPARATIVE RESULTS

In our first report of ESTD, five SESCNs with a mean diameter of 57 mm (range: 40-80 mm) were *en bloc* and R0 resected without any complications[5]. Mean operation duration was 78.6 min (34-120 min) and no recurrence of SESCNs was observed with a mean follow-up of 7.4 mo (3-13 mo), preliminarily showing the feasibility and efficiency of ESTD. Several studies from different centers achieved similar favorable results. Xiong *et al*[19] reported that *en bloc* and R0 resection were achieved in all seven SESCNs treated with ESTD. In another retrospective study of 11 ESTD procedures by Pioche *et al*[17], *en bloc* and R0 resection rate were 100% and 81.8% (2/11), respectively. One patient (9.1%) experienced recurrence. Arantes *et al*[15] treated 25 esophageal neoplasms from 23 patients, ranging from 10 to 60 mm, with ESTD. *En bloc* resection was successfully performed in 23 lesions, and R0 resection was achieved in 22 lesions. Two of 23 patients experienced local recurrence, and one patient underwent reoperation, with supplementary EMR and radiofrequency ablation.

We performed a systematic literature review of ESTD from Chinese databases (CBM, Wanfang Data, CMJD and CNKI) and English databases (PubMed, EMBase and Cochrane Library). The review identified a total of 90 lesions (88 patients) from nine studies, with a mean size of 37.8 mm (range: 10-80 mm) (Table 1). The pooled *en bloc*, R0 resection and local recurrence rates were 97.8% (92%-100%), 85.6% (81.8%-100%), and 3.3% (0%-9.1%), respectively, which were at least comparable to those for ESD. In

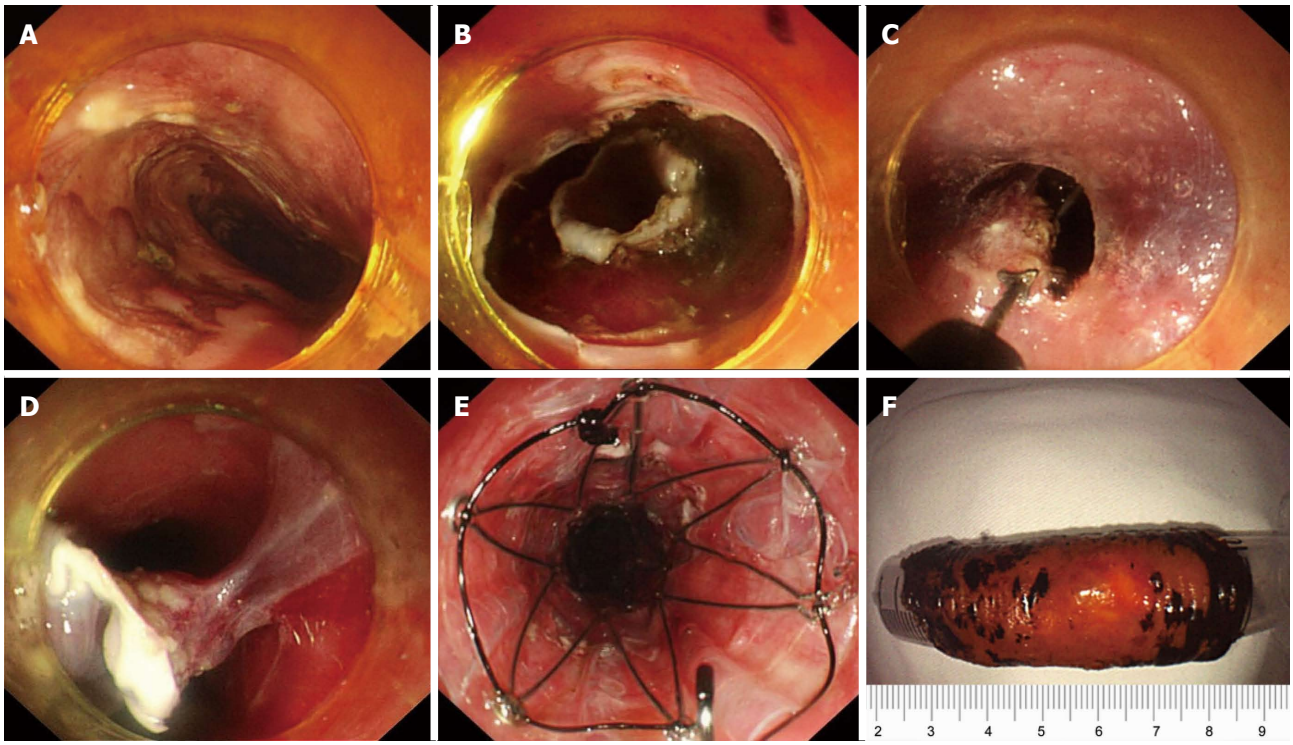

Figure 4 Double-tunnel endoscopic submucosal tunnel dissection for circumferential superficial esophageal neoplasms. A: An 8 cm circumferential superficial esophageal cancer was showed by iodine staining, at 28–36 cm from the incisors; B: Circular incisions were successively performed at the anal and oral margins after marking; C, D: Two submucosal tunnels were created opposite each other. Two tunnels nearly covered the whole esophageal lumen, and the borders were narrow enough to be resected easily; E: A 14 cm retrievable, fully-covered esophageal stent was placed to prevent postoperative stenosis. F: The lesion was resected circumferentially, about 60 mm in length. From Zhai *et al*[20].

Table 1 Systematic literature review of endoscopic submucosal tunnel dissection for superficial esophageal squamous cell neoplasms *n* (%)

Ref.	Year	Lesions/cases	Mean size (mm)	Operation time (min)	*En bloc* resection	R0 resection	Complications	follow-up (mo)	Local recurrence
Linghu *et al*[5] Linghu *et al*[11] Zhai *et al*[32]	2011 2013 2014	11/11	48.2 (30-80)	78.6 (34-120)	100%	81.8%	Stenosis: 6 (54.5)	13.5 (3-30)	0%
Gao *et al*[18]	2012	17/17	24 (20-50)	128 (60-180)	100%	82.4%	Delayed bleeding: 1 (5.9)	NA	0%
Xiong *et al*[19]	2013	7/7	35.7 (20-40)	61 (37-110)	100%	100%	Immediate bleeding: 2 (28.6)	12	0%
Arantes *et al*[15]	2013	25/23	25 (10-60)	85 (60-210)	92%	84%	SE and ME: 2 (8) Perforation: 1 (4)	21.4 (3-36)	2 (8)
Pioche *et al*[17]	2013	11/11	45 (27-80)	76 (35-150)	100%	81.8%	SE: 9.1% Stenosis: 4 (36.4)	NA	1 (9.1)
Tan *et al*[65]	2014	1/1	50	NA	100%	100%	None	6	0%
Zhou *et al*[66]	2014	18/18	58 (45-74)	54.5 (32-85)	100%	100%	Immediate bleeding: 1 (5.6) SE: 1 (5.6) Stenosis: 3 (16.7)	6	0%
Total		90/88	37.8 (10-80)	83.3 (32-180)	88 (97.8)	77 (85.6)	Immediate bleeding: 4 (4.4) Delay bleeding: 1 (1.1) Perforation: 1 (1.1) SE or ME: 3 (3.3) Stenosis: 9 (10)	NA	3 (3.3)

SE: Subcutaneous emphysema; ME: Mediastinal emphesema.

our retrospective comparative study[32], among 29 consecutive patients, 11 patients underwent ESTD and 18 ESD for lesions that were at least one-third the circumference and ⩾ 20 mm in diameter. ESTD had a more rapid dissection speed, almost twice that of ESD (22.4 mm^2/min *vs* 12.2 mm^2/min). Curative resection was more likely to be achieved with ESTD (81.8% *vs* 66.7%). These advantages of ESTD may be attributed to the following factors[5,32]: (1) counter-traction of bilateral mucosae provides a clear submucosal vision for dissection; (2) the transparent cap and gas cushion created by CO_2 insufflation can play a role in blunt

dissection, accelerating the operation; (3) submucosal injection solution is retained longer because ESTD avoids conventional circumferential incision, and there is less need for repeated injection; and (4) because the dissection is deeper in the submucosa, close to the muscularis mucosae, and the mucosa together with most of the submucosa can be removed, making for more precise pathological assessment.

COMPLICATIONS

Bleeding and perforation are the common adverse events of ESTD, with a pooled incidence of 4.4% and 1.1%, respectively. These incidences do not differ significantly from those of ESD, which were reported as 0%-5.2% and 0%-6.9%, respectively[26,33-37]. Submucosal dissection is performed in the deeper submucosa, where transversed vessel trunk prevails. After exposure of the vessel trunk, prophylactic coagulation with hemostatic forceps should be undertaken in soft mode. For minor oozing bleeding, an electrosurgical knife in coagulation mode is usually sufficient, while for massive active bleeding, identification of bleeding spots with a water jet is more advisable than blind coagulation with hemostatic forceps. Clips are reserved for the last resort because they may hinder the next dissection procedure. Unstopped hemorrhage is rare and is mostly caused by delayed bleeding in the first 24 h postoperatively. In 17 patients with ESTD[18], one patient had delayed bleeding and was transferred to surgery after failed endoscopic hemostasis. Proton pump inhibitors are routinely administered, and emergency endoscopy should be performed once delayed bleeding is suspected during close surveillance.

Perforation during operation is usually < 10 mm, and endoscopic clips are the standard method for closing such perforations. Generally, multiple clips are required in a zipper fashion to ensure complete closure. Recently, a new over-the-scope clip has become clinically available and can successfully seal esophageal perforation up to 20 mm in size with high compression force[38-42]. For larger perforations, fully-covered self-expandable metal stents (FCSEMs) are effective, although they have a potential risk of stent migration[43]. For delayed perforation, early recognition determines treatment option and outcome. As a result of minor inflammation and content egression, delayed perforations within 12 h can be treated conservatively, including endoscopic closure with clips and percutaneous drainage. However, surgery is mandatory for patients with unstable hemodynamics, with perforations that are recognized after > 24 h, or who fail conservative treatment[44].

Although one tries to avoid touching the MP during dissection, air-related adverse events frequently occur; partly due to increased air pressure in the submucosal tunnel and thin MP without compact serosa. In 25 ESTDs with room-air insufflation by Arantes *et al*[15], two patients experienced subcutaneous and mediastinal emphysema, while 11 patients reported by us were uneventful with CO_2 insufflation during ESTD[32]. Consensus has been reached that CO_2 insufflation is strongly recommended in DETT[5,10,14,31,45]. The pooled incidence is 4.4% (0%-9.1%), which is lower than that of POEM or STER. All the emphysema was minor and could be absorbed spontaneously without additional treatment.

Postoperative esophageal stricture is of most concern for large mucosal defects after ESTD because it reduces quality of life. Circumference extent and histological depth are reliable predictors of postoperative stricture[23,24,46]. In a retrospective study of 84 esophageal ESDs by Ono *et al*[34], the incidence of lesions less than half, less than three-quarters, and more than three-quarters of the circumference was 2% (1/49), 20% (5/25), and 90% (9/10), respectively. Shi *et al*[24] also reported similar results in a review of 362 ESDs, in which lesions that were more than three-quarters of the circumference had a 93.3% (32/34) risk of stricture. Therefore, preventive intervention should be implemented for lesions that exceed three-quarters of the circumference or are less than three-quarters of the circumference but invade to a greater depth than m2[24,32,47].

Endoscopic pneumatic dilation (EPD) is taken as the standard treatment for postoperative esophageal strictures[48,49]. However, refractory strictures caused by large defects usually demand up to dozens of EPDs. This increases the risk of perforation as well as the treatment cost. In a study of 121 patients with 1337 EPD procedures for post-ESD stenosis[50], the incidence of perforation was 4.1% (5/121) per patient and 0.4% (5/1337) per procedure. Patients with perforations required more sessions of EPD than those without perforations [37 times (6-57) *vs* 7 times (1-70)]. Takahashi *et al*[51] also reported that seven patients developed perforation during 648 dilation procedures for post-EMR/ESD stricture, and multiple dilations and lower esophageal site were independent risk factors for perforation. Systemic administration or local endoscopic injection of steroid is effective, which not only lowers incidence of stricture, but also decreases extension of stricture and numbers of required endoscopic dilations[52-56]. In a recent randomized controlled trial by Takahashi *et al*[57], 32 patients with post-ESD defects more than three-quarters of the circumference were randomized to single triamcinolone acetonide injection (n = 16) and conventional EPD (n = 16). The steroid group required fewer dilatation sessions than the control group (6.1 sessions *vs* 11.5 sessions), without an increased risk of perforation. With respect to circumferential ESD, Sato *et al*[56] reported that oral steroid therapy with EPD was more effective than single EPD for the prevention of esophageal stricture. Given possible multiple local injections and mistaken deep injection into the MP, convenient oral steroid therapy with EBD

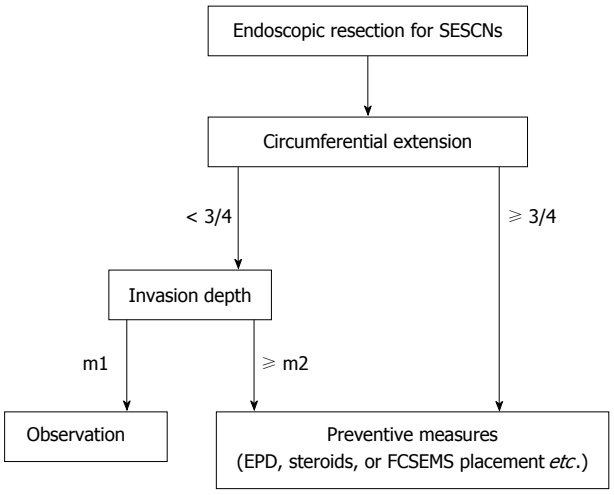

Figure 5 Proposed flow diagram of postoperative stricture prevention for superficial esophageal squamous cell neoplasms after endoscopic resection.

on demand may be a preferred option for prevention and treatment of post-ESD stricture, in spite of a lack of studies comparing oral and local steroid injection. In our endoscopy center, FCSEMs are routinely placed for lesions more than three-quarters of the circumference with a period of 4-8 wk, which has been shown to decrease incidence of stricture and reduce the need for dilation[58]. Therefore, we suggest an algorithm for the prevention of postoperative stricture after endoscopic resection of SESCNs (Figure 5). Recently, other approaches, such as bioabsorbable poly-L-lactic acid stent placement[59], polyglycolic acid sheet with fibrin glue[60], and transplantation of tissue-engineered cell sheets[47,61], have shown promising results, but these approaches require further studies with large samples prior to extensive clinical use.

PERSPECTIVES AND OTHER APPLICATIONS

ESTD has established a new strategy for large SESCNs, which is different from that of traditional ESD, namely marking-circumferential incision-submucosal dissection. ESTD is derived from submucosal dissection techniques and advances in endoscopic equipment. As an important branch of DETT, ESTD is another good reflection of the submucosal tunnel concept, like popular POEM and STER. Compared with the latter procedures, ESTD does not have any operative difficulties and may be safer due to maintaining the MP layer untouched. Hence, we believe that endoscopists with experience of POEM or STER will find it easy to adopt ESTD, along with its parallel learning curve and training. Arantes *et al*[15] described their ESTD learning process, which involved several sessions of hands-on practice on animals; experience as first-assistant in human procedures; unsupervised ESD operations for gastric and rectal tumors (25 cases); and ESTD operations. With the excellent performance in 25 patients, Arantes *et al*[15] proposed ESTD as a standardized approach to facilitate ESD learning for Western endoscopists. Moreover, the promising results from both Asian and Western endoscopy centers and the advantages of ESTD over conventional ESD enable us to speculate that ESTD will replace ESD as a standardized method for large SESCNs. A multicenter prospective study of ESTD by us is ongoing, and other large-sample research from different centers is awaited. A prospective randomized study comparing ESTD with ESD is also eagerly anticipated.

ESTD can be also used for superficial lesions in other parts of the digestive tract. We have reported initial experience of ESTD for gastric lesions (three in the cardia and one in the lesser curvature), which were successfully removed in *en bloc* and R0 fashion without any complications[62]. The mean diameter was 43 mm (40-50 mm), and the mean operation time was 65 min (34-97 min). However, it is easy to miss lesions in the gastric tunnel by submucosal dissection, because the lesions are not always in a straight line, and superficial marks are invisible from the tunnel view. Frequent withdrawal of the endoscope from the tunnel is necessary. To overcome the limitations of submucosal fibrosis, Choi *et al*[63] utilized ESTD as a salvage technique to treat two patients with ulcerative early gastric cancer with fibrosis. The salvage technique made some modifications, by which the oral incision was 2-3 cm distal to lesions, and the anal was cut open in an arcuate style. To resect a large sessile duodenal adenoma in the bulb adjacent to a scar, Jin *et al*[16] created a submucosal tunnel through the pyloric ring for its removal. *En bloc* resection was achieved, and two minor perforations during hemostasis were completely clipped after submucosal dissection. The postprocedural course was uneventful, and the patient was discharged 8 d after ESTD. Hu *et al*[64] reported successful application of STER to resect eight rectal submucosal tumors. Despite absence of clinical use of ESTD in the colorectum, we still believe it is feasible to remove large, laterally spreading tumors from the rectum and distal colon. In our experience, given different anatomical features and potential risk, ESTD is currently indicated for lesions limited to the gastric cardia, lesser curvature of the gastric body, antrum, and rectum.

CONCLUSION

In conclusion, as a new treatment strategy, ESTD is an effective and safe method for large SESCNs. ESTD can thoroughly remove the obstacle of endoscopic resection due to lesion size and is recommended for lesions that do not invade deeper than sm1 and without clinical evidence of lymph node metastasis and at least one-third of the circumference of the esophagus and ⩾ 20 mm in diameter. Postoperative esophageal stricture has received much attention, and preventive

intervention should be implemented for lesions that exceed three quarters of the circumference, or lesions that are less than three-quarters of the circumference but invading deeper than m2. Although studies to date have been limited and larger studies with a high level of evidence are required, the promising results so far enable us to speculate that ESTD will replace traditional ESD as the standard treatment for large SESCNs in the near future.

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SYSTEMATIC REVIEWS

Distinctive aspects of peptic ulcer disease, Dieulafoy's lesion, and Mallory-Weiss syndrome in patients with advanced alcoholic liver disease or cirrhosis

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Abstract

AIM: To systematically review the data on distinctive aspects of peptic ulcer disease (PUD), Dieulafoy's lesion (DL), and Mallory-Weiss syndrome (MWS) in patients with advanced alcoholic liver disease (aALD), including alcoholic hepatitis or alcoholic cirrhosis.

METHODS: Computerized literature search performed *via* PubMed using the following medical subject heading terms and keywords: "alcoholic liver disease", "alcoholic hepatitis"," alcoholic cirrhosis", "cirrhosis", "liver disease", "upper gastrointestinal bleeding", "non-variceal upper gastrointestinal bleeding", "PUD", "DL", "Mallory-Weiss tear", and "MWS".

RESULTS: While the majority of acute gastrointestinal (GI) bleeding with aALD is related to portal hypertension, about 30%-40% of acute GI bleeding in patients with aALD is unrelated to portal hypertension. Such bleeding constitutes an important complication of aALD because of its frequency, severity, and associated mortality. Patients with cirrhosis have a markedly increased risk of PUD, which further increases with the

progression of cirrhosis. Patients with cirrhosis or aALD and peptic ulcer bleeding (PUB) have worse clinical outcomes than other patients with PUB, including uncontrolled bleeding, rebleeding, and mortality. Alcohol consumption, nonsteroidal anti-inflammatory drug use, and portal hypertension may have a pathogenic role in the development of PUD in patients with aALD. Limited data suggest that *Helicobacter pylori* does not play a significant role in the pathogenesis of PUD in most cirrhotic patients. The frequency of bleeding from DL appears to be increased in patients with aALD. DL may be associated with an especially high mortality in these patients. MWS is strongly associated with heavy alcohol consumption from binge drinking or chronic alcoholism, and is associated with aALD. Patients with aALD have more severe MWS bleeding and are more likely to rebleed when compared to non-cirrhotics. Pre-endoscopic management of acute GI bleeding in patients with aALD unrelated to portal hypertension is similar to the management of aALD patients with GI bleeding from portal hypertension, because clinical distinction before endoscopy is difficult. Most patients require intensive care unit admission and attention to avoid over-transfusion, to correct electrolyte abnormalities and coagulopathies, and to administer antibiotic prophylaxis. Alcoholics should receive thiamine and be closely monitored for symptoms of alcohol withdrawal. Prompt endoscopy, after initial resuscitation, is essential to diagnose and appropriately treat these patients. Generally, the same endoscopic hemostatic techniques are used in patients bleeding from PUD, DL, or MWS in patients with aALD as in the general population.

CONCLUSION: Nonvariceal upper GI bleeding in patients with aALD has clinically important differences from that in the general population without aALD, including: more frequent and more severe bleeding from PUD, DL, or MWS.

Key words: Alcoholic liver disease; Alcoholic hepatitis; Cirrhosis; Portal hypertension; Peptic ulcer disease; Mallory-Weiss syndrome; Dieulafoy lesion; Endoscopic therapy

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Core tip: Alcoholism is highly prevalent worldwide and can cause advanced-alcoholic-liver-disease (aALD) from alcoholic hepatitis or cirrhosis. This work systematically reviews the literature on acute-upper-gastrointestinal-bleeding not directly related to portal hypertension in patients with aALD. Such patients have markedly increased risks of peptic ulcers, and worse outcomes from peptic ulcer bleeding than other patients, including refractory bleeding, rebleeding, and mortality. Such patients apparently have increased frequency and mortality of bleeding from Dieulafoy lesions. Such patients have more frequent, more severe, and more rebleeding from Mallory-Weiss-syndrome than non-cirrhotics. Prompt endoscopy, after resuscitation, is essential to diagnose and appropriately treat these patients, using endoscopic therapy when necessary.

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INTRODUCTION

Alcohol consumption remains highly prevalent worldwide despite its association with more than 60 diseases as well as accidental injuries[1]. For example, about two-thirds of adult Americans consume some alcohol[2], and up to one-sixth are alcoholics[3]. Although the burden of alcohol-related disease is generally highest in developed countries, the burden is also large and likely increasing in developing countries[4].

Chronic excessive alcohol consumption can cause liver injury, ranging from alcoholic fatty liver disease to alcoholic hepatitis to alcoholic cirrhosis[5]. These entities can occur simultaneously in one individual (*e.g.*, alcoholic hepatitis with alcoholic cirrhosis)[6]. Alcoholic cirrhosis, the least common but most severe form of alcoholic liver disease, occurs in approximately 15% of individuals chronically abusing alcohol[7]. Alcoholic cirrhosis is associated with numerous life-threatening complications, including variceal bleeding, spontaneous bacterial peritonitis, hepatic encephalopathy, hepatorenal syndrome, and hepatocellular carcinoma[8].

Alcoholic hepatitis and alcoholic cirrhosis are herein denoted as advanced alcoholic liver disease (aALD). Upper gastrointestinal (UGI) bleeding in patients with aALD is clinically important due to its frequency, severity, and high mortality[9,10]. UGI bleeding accounts for up to 25% of the mortality from cirrhosis[11], and up to 10% of the mortality from alcoholic hepatitis[12]. Table 1 describes etiologies of UGI bleeding associated with aALD or cirrhosis, but excludes etiologies not believed to be associated with cirrhosis, such as reflux esophagitis, duodenitis, GI tumors, and angiodysplasia[13-31]. UGI bleeding related to cirrhosis or aALD includes etiologies directly related *vs* unrelated to portal hypertension. Etiologies directly related to portal hypertension include esophageal varices[32], gastric varices[15], duodenal varices[16], gastric portal hypertensive gastropathy[33], and gastric antral vascular ectasia (GAVE)[34]. Also, cirrhotic patients rarely develop lower GI bleeding related to portal hypertension from rectal varices[35]. Variceal bleeding has been frequently reviewed because it represents the etiology of UGI bleeding in about 70% of cases in cirrhotic patients, occurs in 25%-40% of cirrhotics, and constitutes a major cause of their mortality[36].

Table 1 Differential diagnosis of acute upper gastrointestinal bleeding in an alcoholic with advanced liver disease

Bleeding lesion	Endoscopic appearance	Pathophysiology	Nonsurgical treatment	Ref.
Etiologies related to portal hypertension				
Esophageal varices	Serpiginous, bluish-grey, vessels protruding from the mucosa into the lumen that typically are largest just above the gastroesophageal junction	Varices provide a conduit for venous blood blocked from traversing through the liver because of cirrhosis to return to the heart	Endoscopy: variceal banding, variceal sclerotherapy Angiography: TIPS Other: balloon tamponade, octreotide	Garcia-Tsao *et al*[13], Beppu *et al*[14]
Gastric varices	Serpiginous, bluish-grey, vessels protruding from the mucosa into the lumen that are most commonly located in the gastric cardia, fundus or body	Varices provide a conduit for venous blood blocked from traversing through the liver because of cirrhosis to return to the heart Formation of gastric varices may be promoted by endoscopic obliteration of esophageal varices	Angiography: TIPS Other: balloon tamponade, octreotide Endoscopy: cyanoacrylate injection therapy	Garcia-Pagán *et al*[15]
Duodenal varices	Resemble esophageal varices in endoscopic appearance, but are located within duodenum	Rare site of varices which may be promoted by prior esophageal variceal banding or sclerotherapy and prior duodenal surgery	Endoscopy: variceal banding or sclerotherapy Angiography: TIPS, angiographic occlusion therapy by embolization or balloon occlusion	Copelan *et al*[16], Matsui *et al*[17]
Portal hypertensive gastropathy	Mosaic or snake-skin appearance of gastric mucosa, especially of the gastric fundus and proximal gastric body, due to dilated, ectatic, superficial mucosal vessels	Network of microcirculation that drains venous blood blocked from passing through the cirrhotic liver to return to the left atrium	TIPS	Patwardhan *et al*[18], Thuluvath *et al*[19]
Etiologies possibly related to portal hypertension				
Gastric antral vascular ectasia	Intensely erythematous streaks on longitudinal folds oriented towards the pylorus in the antrum	May be related to stretch of antral vessels from duodenal bulb prolapse. Vascular engorgement from portal hypertension or from hormonal abnormalities (*e.g.*, hyperestrogenemia with cirrhosis) may also contribute to lesion pathogenesis	Endoscopic therapy: APC, thermocoagulation, electrocoagulation, or radiofrequency ablation.	McGorisk *et al*[20], Payen *et al*[21]
Etiologies possibly related to alcoholism or advanced liver disease				
Peptic ulcer disease	Focal ulcer: (depressed) crater covered by mucopurulent material	Major causes in general population include *H. pylori* infection or NSAID use. Idiopathic PUD is increasingly noted. Gastric infections or gastric malignancy may mimic PUD. Pathogenesis of PUD in ALD and cirrhosis discussed in text	Endoscopic therapy: discussed in text	Siringo *et al*[22], Vergara *et al*[23], Kamalaporn *et al*[24], D'Amico *et al*[25]
Mallory-Weiss tear	Longitudinally oriented erythematous tear or crack in the mucosa that straddles the gastroesophageal junction	Laceration due to mucosal trauma from retching or vomiting. Frequently associated with binge drinking or chronic alcoholism	Endoscopic therapy: discussed in text	Paquet *et al*[26], Schuman *et al*[27], Jensen *et al*[28]
Dieulafoy's lesion	Elevated, pigmented spot that projects into the lumen from the mucosal surface without surrounding ulceration	Caliber-persistent artery near mucosal surface can erupt through thin overlying mucosal cells and cause bleeding	Endoscopic therapy: injection therapy, band ligation, electrocoagulation, APC Angiography: local embolization Surgery: wedge resection	Cappell[29], Jeon *et al*[30], Nojkov *et al*[31]

TIPS: Transjugular intrahepatic portosystemic shunt; APC: Argon plasma coagulation; PUD: Peptic ulcer disease; *H. pylori*: *Helicobacter pylori*; ALD: Alcoholic liver disease; NSAID: Nonsteroidal anti-inflammatory drug.

The relative frequency of acute UGI bleeding, not directly related to varices or portal hypertension, is estimated at 30%-40% of UGI bleeding in cirrhotic patients[10,37-40]. Peptic ulcer disease (PUD), Dieulafoy's lesion (DL), and Mallory-Weiss syndrome (MWS) are strongly associated with alcoholic cirrhosis or cirrhosis in general[41,42]. These bleeding etiologies may be related to alcohol because alcohol can damage the gastric mucosal barrier, stimulate gastric acid secretion, and cause nausea and vomiting that induces MWS[43]. This review comprehensively and critically analyzes the association between PUD, DL, and MWS and aALD or cirrhosis; demonstrates underappreciated, major differences in the pathophysiology and natural history of these bleeding etiologies in cirrhotic patients compared to non-cirrhotic patients; and discusses the clinical consequences in terms of treatment and prognosis.

MATERIALS AND METHODS

Computerized search of the literature was performed *via* PubMed using the following medical subject heading

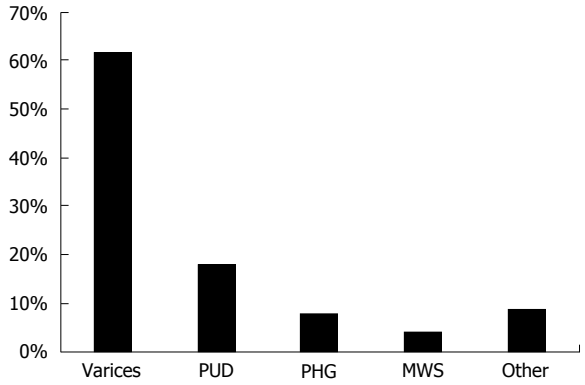

Figure 1 Reported distribution of common etiologies of upper gastrointestinal bleeding in patients with cirrhosis. Frequency distribution of the etiologies of upper gastrointestinal bleeding in patients with cirrhosis. Varices include esophageal or gastric varices. Adapted from Ref. [39,44-47]. PUD: Peptic ulcer disease; PHG: Portal hypertensive gastropathy; MWS: Mallory-Weiss syndrome; Other: Other etiologies of upper gastrointestinal bleeding.

(MeSH) terms and keywords: "alcoholic liver disease", "alcoholic hepatitis"," alcoholic cirrhosis", "cirrhosis", "liver disease", "upper gastrointestinal bleeding", "non-variceal upper gastrointestinal bleeding", "PUD", "DL", "Mallory-Weiss tear", and "MWS". Of about 1200 articles initially identified by computerized review, about 825 articles were eliminated as not relevant to the subject of this paper after briefly reviewing the articles, including thoroughly reviewing the abstracts. The remaining 375 articles were thoroughly reviewed, and 165 articles were selected for incorporation and citation in this systematic review. The following criteria were applied for study inclusion: publication in peer-reviewed journals, studies published since 1980, reporting outcomes for non-variceal upper GI bleeding in patients with underlying liver disease, and reporting the etiology of liver disease. Criteria for prioritizing publications for review inclusion included: well-designed, prospective trials; recent studies; large study populations; and study emphasis on aALD. However, data from retrospective series, reviews from internationally recognized authorities, and even case reports were included when prospective trials were unavailable. The authors thank Jadranka Stojanovska, M.D., M.S., Assistant Professor of Radiology, University of Michigan Health System, Ann Arbor, Michigan, for her expert biostatistical review of the manuscript.

RESULTS

Nonvariceal bleeding in cirrhotics

The estimated distribution of common etiologies of acute upper GI bleeding, unrelated to portal hypertension, in patients with cirrhosis are shown in Figure 1[39,44-47].

PUD

Epidemiology: Patients with cirrhosis have a significantly increased prevalence of PUD, with a reported point prevalence of 10% or higher[22-24,48,49]. For example, among the 324 of 368 consecutive cirrhotic patients who underwent endoscopic screening for esophageal varices, 11.7% were diagnosed with PUD[22]. The annual incidence of developing new PUD was 4.3% during endoscopic follow-up among 140 of these cirrhotic patients, an annual incidence that is much higher than that of PUD in the general population of < 0.5%[22]. In a prospective study of 130 patients with cirrhosis undergoing esophagogastroduodenoscopy (EGD) for variceal screening, 50 patients (39%) had PUD[24]. There were no significant differences in age, sex, or history of smoking tobacco between patients with *vs* without PUD. In a prospective South Korean study, PUD was found in 24.3% of the 288 patients with cirrhosis[50].

Advanced cirrhosis (Child-Pugh class B or C) is particularly associated with PUD[24]. For example, among 324 patients undergoing EGD, patients with advanced cirrhosis had a significantly higher frequency of PUD than patients with early cirrhosis ($P = 0.04$)[22].

PUD is the most common cause of nonvariceal UGI bleeding in patients with cirrhosis[25,46,51]. Among 160 patients with non-variceal UGI bleeding and cirrhosis (mostly from alcohol), 81 patients (50.6%) had PUD, and 53.1% had high-risk endoscopic stigmata of recent hemorrhage (SRH) at the ulcer base[52]. The reported prevalence of acute UGI bleeding from PUD (PUB) in patients with chronic liver disease ranges from 1.6% to 25%[22,47,53-55]. This wide variability likely reflects the diverse patient populations from different countries and different socioeconomic groups among various studies, and study of patients at different stages of liver disease. A recent, nationwide database study from Taiwan, conducted over 7 years, showed that PUB was significantly more prevalent among 4013 patients with nonalcoholic cirrhosis (4.8%), compared to either 8013 patients with chronic hepatitis without cirrhosis (1.6%) or 7793 controls without liver disease (1.6%)[53]. The three patient groups were matched for age, sex, comorbidities, medications, and annual income. This difference remained statistically significant even after adjusting for confounding factors (HR = 4.22; 95%CI: 3.37-5.29, $P < 0.001$)[53]. Advanced age, male sex, diabetes mellitus, chronic renal disease, prior variceal bleeding, and use of nonsteroidal anti-inflammatory drugs (NSAIDs) were risk factors for PUB in the cirrhotic patients.

Clinical outcomes: Patients with PUD associated with cirrhosis have higher rates of bleeding complications, of delayed ulcer healing, and of ulcer recurrence as compared to the general population[22,56]. For example, Siringo *et al*[22] showed that gastric ulcers healed more slowly and recurred more frequently than gastric ulcers in controls without cirrhosis ($P = 0.04$). The bleeding may be exacerbated by thrombocytopenia or an elevated international normalized ratio (INR) associated with cirrhosis and portal hypertension. Siringo *et al*[22] further reported that 79% of peptic

Table 2 Clinical characteristics of patients with cirrhosis presenting with upper gastrointestinal bleeding unrelated to portal hypertension predominantly from peptic ulcers

Ref.	No. of parents	ALD (%)	Mean age	Prevalence of PUB (%)	Mortality (%)	Predictors of mortality	Rebleeding rate (%)	Length of follow-up
González-González *et al*[52]	160	"Most"	56.5 ± 14.4	50.6	13.8	High BUN, Low albumin, Active ulcer bleeding, Need for endoscopic therapy, Cryptogenic cirrhosis	1.9	NA
Seo *et al*[61]	107	43	55.8 ± 11	60	14.5	Failure to control bleeding, High creatinine, High AST, High Child-Pugh score, PVT	9.3	6 wk
Morsy *et al*[62]	93	NA	55.3 ± 11.2	30	14	Bacterial infection, Shock, Early rebleeding, Low albumin, Low hemoglobin, Endoscopic therapy	4.3	Short-term (in-hospital)
Siringo *et al*[22]	85	40	62 ± 12	41	23[1]	Low hemoglobin, Encephalopathy Alcoholism, High bilirubin, PVT, High Child-Pugh score, High AST/ALT levels, HCC	0	6 wk
Rudler *et al*[63]	29	24 (83)	55.0 ± 9.0	29 (100)[2]	1 (3)	NA	2 (7)	30 d

[1]Mortality from all etiologies of non-variceal GI bleeding; [2]PUD presence - study inclusion criteria. BUN: Blood urea nitrogen; PVT: Portal vein thrombosis; AST/ALT: Aspartate aminotransferase/alanine aminotransferase; HCC: Hepatocellular carcinoma; NA: Not available.

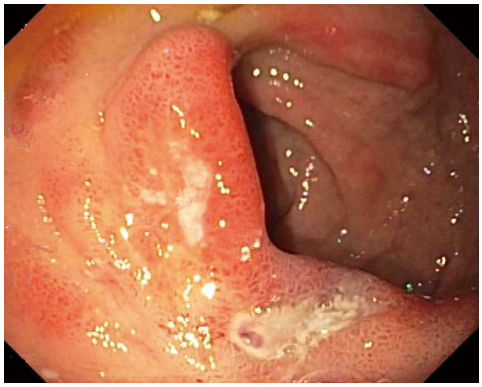

Figure 2 Seventy-year-old male with Child-Pugh class A alcoholic cirrhosis presented with hematemesis, melena, and orthostatic dizziness. Esophagogastroduodenoscopy (EGD) revealed an acute 14 mm × 5 mm ulcer in the distal duodenal bulb with stigma of recent hemorrhage (SRH) represented by a prominent, nonbleeding, flat, pigmented spot; trace esophageal varies with no SRH, such as wale bites; and no portal hypertensive gastropathy. Note the deep erosion adjacent to the ulcer. The etiology of the ulcer was idiopathic. The patient had no history of taking nonsteroidal anti-inflammatory drugs or aspirin. Pathologic examination of gastric biopsies taken at EGD revealed no *Helicobacter pylori*. No endoscopic therapy was performed because the only SRH was a flat pigmented spot which has a low risk of rebleeding. The patient, however, required transfusion of 4 units of packed erythrocytes. The patient was treated medically with pantoprazole with no recurrence of the bleeding. This patient illustrates that peptic ulcers are in the differential of acute upper gastrointestinal bleeding in a patient with cirrhosis, even in the absence of risk factor for peptic ulcers, and that bleeding from peptic ulcers in cirrhotic patients may be unusually severe.

ulcer recurrences in patients with cirrhosis were asymptomatic. This emphasizes the clinical importance of endoscopic follow-up of gastric ulcers in this population. In a recent, nationwide, database analysis from Taiwan of patients hospitalized for PUB from 1997-2006, patients with cirrhosis had a much higher rate of rebleeding from PUD than patients without cirrhosis (HR = 3.19; 95%CI: 2.62-3.88)[57].

In a recent, retrospective, Finnish study of 518 patients with a first episode of nonvariceal UGI bleeding (NVUGIB), the rebleeding rate at one year among 102 alcoholics was significantly higher at 16.7% than the rate among the 416 nonalcoholic patients of 9.1% (P = 0.027)[58]. About half of the patients bled from PUD in both groups. Figure 2 illustrates the endoscopic findings of an acute 14 mm × 5 mm peptic duodenal ulcer with low risk stigmata of recent hemorrhage (SRH), consisting of a nonbleeding, flat, pigmented spot, in a patient with Child-Pugh class A alcoholic cirrhosis experiencing upper GI bleeding. Despite the absence of high risk SRH, the patient required transfusion of 4 units of packed erythrocytes for the bleeding episode.

Patients with advanced liver disease have increased mortality from PUB. In the aforementioned study from Taiwan, the 9711 patients with cirrhosis had significantly higher mortality-adjusted rebleeding rates than the 38844 matched controls without cirrhosis at 1 year (14.4% *vs* 11.3%, P < 0.001), 5 years (26.1% *vs* 22.5%, P < 0.001), and 10 years (28.4% *vs* 27.1%, P < 0.001)[57]. A population-based study of 21359 patients hospitalized in Denmark from 2004-2011 included 1127 (5.3%) with chronic liver disease, of whom 3.1% had cirrhosis and 2.2% did not have cirrhosis[59]. Cumulative 90-d mortality after discharge was significantly higher in patients with chronic liver disease: 25% mortality in patients with cirrhotic and 21% mortality in patients with chronic liver disease without cirrhosis *vs* 18% mortality in patients without chronic liver disease. After adjusting for confounding variables, including comorbidities, patients with cirrhosis had more than twice the mortality of patients without liver disease (OR = 2.38, 95%CI: 2.02-2.80), and patients with liver disease without cirrhosis had 46% higher mortality than patients without liver disease. In a cross-sectional, nationwide, study from the United States of 96887 inpatients admitted for PUB in 2009, the 3574 patients (3.7%) with cirrhosis had significantly higher mortality from PUB than patients

Table 3 Key clinical findings regarding peptic ulcer disease in patients with alcoholic cirrhosis

Key finding	Ref.
Increased prevalence of PUD in patients with cirrhosis. Most common etiology of non-variceal hemorrhage in cirrhotics	Siringo *et al*[22], Vergara *et al*[23], Kamalaporn *et al*[24], D'Amico *et al*[25], Lo *et al*[48], Rabinovitz *et al*[49]
Advanced cirrhosis (Child-Pugh stage C) more strongly associated with PUD than early cirrhosis (Child-Pugh stage A)	Kamalaporn *et al*[24]
Most patients with PUD associated with cirrhosis are asymptomatic	Siringo *et al*[22]
Patients with cirrhosis are more likely to bleed from PUD than other patients with PUD	Luo *et al*[53]
Higher frequency of complications from bleeding PUD in patients with cirrhosis	Siringo *et al*[22], Di Mario *et al*[56]
Higher rate of re-bleeding from PUD in cirrhotics	Hsu *et al*[57]
Alcohol impairs ulcer healing and decreases patient compliance with anti-ulcer therapy	Reynolds *et al*[64]
Higher mortality from PUB in cirrhotics compared to non-cirrhotics	Holland-Bill *et al*[59], Venkatesh *et al*[60]
Cirrhotic patients with PUD do not have a higher rate of *H. pylori* infection than non-cirrhotics with PUD	Kim *et al*[50], Auroux *et al*[65]

PUD: Peptic ulcer disease; PUB: Peptic ulcer bleeding; *H. pylori*: *Helicobacter pylori*.

without cirrhosis (5.5% *vs* 2.0%, $P < 0.01$), with an even higher mortality of 6.6% in decompensated cirrhotics[60]. The investigators reported no differences in endoscopic utilization between patients with *vs* without cirrhosis, but patients with cirrhosis incurred higher hospitalization costs, despite less frequently undergoing surgery for PUB.

Although patients with PUB and cirrhosis have worse clinical outcomes than patients with only PUB, they still have better clinical outcomes than patients with acute variceal bleeding and cirrhosis. A large, multicenter, prospective cohort study of 465 Italian patients with cirrhosis and acute UGI hemorrhage reported that 25% had non-variceal bleeding, most commonly from PUD[25]. Compared to patients with variceal bleeding, patients with PUB had significantly lower risks of uncontrolled initial bleeding (7% *vs* 15%), rebleeding (9.6% *vs* 19%), and mortality (15% *vs* 21%). Risk factors for mortality included alcoholism, serum hyperbilirubinemia, hypoalbuminemia, and presence of hepatic encephalopathy or hepatocellular carcinoma. Predictors of short-term ($\leqslant$ 5 d) unfavorable outcomes of uncontrolled bleeding, rebleeding, or mortality, for patients with either variceal or nonvariceal bleeding, included active bleeding at EGD, low initial hematocrit, elevated aminotransferase levels, advanced Child-Pugh class, and portal vein thrombosis.

Table 2 summarizes data on UGI bleeding, unrelated to portal hypertension, with a focus on PUB in patients with cirrhosis[22,52,61-63]. The data on PUB in Table 2 are somewhat limited by including other etiologies of nonvariceal UGI bleeding aside from PUD, but all listed studies included a majority of cirrhotic patients bleeding from PUD. Table 3 summarizes the key reported clinical findings regarding PUD in patients with aALD or cirrhosis[22-25,48-50,53,56,57,59,60,64,65].

Pathogenesis: The pathophysiology of the association between PUD and cirrhosis is incompletely understood. Alcohol toxicity, portal hypertension, *Helicobacter pylori* (*H. pylori*) infection, and nonsteroidal anti-inflammatory drugs (NSAIDs) have been investigated as etiologic factors.

Patients with aALD have a much higher prevalence of PUD than the general population, and likely have an even higher prevalence of PUD than patients with non-alcoholic cirrhosis[49,65]. This finding is somewhat controversial. In a prospective study of 216 male cirrhotic patients, evaluated for liver transplantation at the University of Pittsburgh, 12.2% of patients with alcoholic cirrhosis had duodenal ulcers, a significantly higher rate of duodenal ulcers than that in patients with non-alcoholic cirrhosis (*e.g.*, cryptogenic, autoimmune, or viral cirrhosis)[49]. Additionally, an alcoholic etiology for cirrhosis is an important predictor of poor outcome 6 wk after acute UGI bleeding, regardless of the bleeding etiology[25]. Furthermore, a recent, prospective, French study reported that 27 of 29 cirrhotics with severe PUB were alcoholics, and that 93% of them had endoscopic evidence of portal hypertension[63]. The clinical outcome from PUB in this study was, surprisingly, not significantly different between patients with cirrhosis *vs* those without cirrhosis, when assessed for rebleeding rate (7% *vs* 6.9%), need for arterial embolization (10.3% *vs* 8.6%), need for salvage surgery (0% *vs* 1.7%), and mortality (3% *vs* 1.1%). However, another study reported similar rates of PUD in alcoholic *vs* nonalcoholic cirrhotics[24].

Alcohol impairs ulcer healing and alcoholism decreases patient compliance with recommended therapies[64]. It is toxic to gastric mucosa, and stimulates gastric acid secretion[43]. However, these factors do not completely explain the increased prevalence of PUD with aALD[66,67].

Data on the role of portal hypertension in PUD pathogenesis in patients with advanced liver disease are somewhat limited and relatively contradictory. Defensive mucosal factors, including gastric mucosal perfusion and mucosal secretion, are impaired in the presence of portal hypertension[68]. Furthermore, reduction of portal hypertension with propranolol reduces alcohol-induced gastric mucosal injury in rats with portal hypertension, and improves the endoscopic appearance of portal

hypertensive gastropathy in patients with cirrhosis, suggesting that portal hypertension may promote PUD by impairing mucosal defenses[68,69].

In a recent, retrospective, single-center study from South Korea of 455 patients with cirrhosis or chronic hepatitis, cirrhosis was more strongly associated with PUD than chronic hepatitis (OR = 4.13, P < 0.03), but an elevated hepatic vein pressure gradient (HVPG) was not a risk factor for PUD in either group of patients[70]. However, another study of 245 cirrhotic patients *vs* 245 healthy controls, matched for age and sex, revealed that an HVPG ⩾ 12 was a significant predictive factor for gastric ulcers in cirrhotic patients (24.4%), as compared to either cirrhotic patients with an HVPG < 12 mmHg (4.5%) or controls without cirrhosis (4.0%)[71]. This study, however, excluded patients with duodenal ulcers. Other studies reported an increased prevalence of endoscopic stigmata of portal hypertension, such as esophageal varices, in cirrhotic patients with PUB, as compared to cirrhotic patients without PUB[63,72]. For example, 27 of 29 patients with cirrhosis and severe PUB had endoscopic features of portal hypertension, *vs* none of 179 patients without cirrhosis and severe PUB had these endoscopic features (P < 0.0001)[63].

In the general population *H. pylori* is a major risk factor for PUD, especially for duodenal ulcers (DUs)[73]. The role of *H. pylori* in the pathogenesis of PUD in patients with advanced liver disease is somewhat controversial, and *H. pylori* likely plays a limited role in such patients. The prevalence of *H. pylori* infection in patients with cirrhosis reportedly ranges widely from 35%-89%, attributed to differences of *H. pylori* prevalence in different countries and in different populations within individual countries, and to the use of different diagnostic tests[50,74-79]. Populations in heavily industrialized countries have a much lower prevalence of *H. pylori* infection[80], while wealthy patients in heavily industrialized countries have the lowest prevalence[81]. Also, studies using serologic testing generally report a higher prevalence of *H. pylori* infection in patients with cirrhosis compared to studies using pathologic examination of gastric biopsies, partly due to the lower positive predictive value of serologic testing[76]. A recent meta-analysis of 21 studies involving 6135 patients, reported a significant association between cirrhosis and *H. pylori* infection in patients with PUD (OR = 2.05, 95%CI: 1.33-3.18, P < 0.0001)[82]. However, subgroup analysis revealed significantly higher prevalence of *H. pylori* infection in patients from Europe (OR = 2.98, 95%CI: 2.02-4.39, P < 0.0001) or from America (OR = 4.75, 95%CI: 1.42-15.95, P = 0.025), but not in patients from Asia (OR = 0.90, 95%CI: 0.48-1.66, not significant).

In a prospective South Korean study in which *H. pylori* infection was diagnosed by rapid urease testing and histologic analysis of gastric biopsies, the prevalence of *H. pylori* infection was significantly lower among 288 patients with cirrhosis (35%), than among either 322 patients with non-ulcer dyspepsia (62.4%), or 339 patients with PUD without cirrhosis (73.7%) (P = 0.001)[50]. Moreover, the prevalence of PUD in patients with Child-Pugh class C cirrhosis was significantly higher than in patients with Child-Pugh class A or B cirrhosis (31% *vs* 21%, P < 0.05), despite patients with Child-Pugh class C cirrhosis having a lower prevalence of *H. pylori* infection.

Also, patients with alcoholic cirrhosis had a significantly lower prevalence of *H. pylori* infection than patients with cirrhosis from viral hepatitis (22% *vs* 42%, P < 0.001), or cryptogenic cirrhosis (22% *vs* 40%, P < 0.001)[50]. Similarly, in a prospective study of 66 cirrhotic patients, including 44 patients with alcoholic cirrhosis, PUD was significantly associated with recent alcohol intake (< 1 wk before EGD) or portal hypertensive gastropathy, but was not significantly associated with *H. pylori* infection[65]. Patients with alcoholic cirrhosis had a lower prevalence of *H. pylori* infection (OR = 0.77, 95%CI: 0.04-16.59, P < 0.0001), than patients with other etiologies of liver disease, including primary biliary cirrhosis (OR = 1.75, 95%CI: 1.15-2.64, P = 0.147), or viral cirrhosis (OR = 2.66, 95%CI: 1.24-5.71, P < 0.0001)[82].

Cirrhotic patients with PUD generally show little or no benefit from *H. pylori* eradication in preventing PUD recurrence[49,83,84]. In a prospective study of 104 patients with cirrhosis and duodenal ulcer (DU), 54 (52%) had *H. pylori* infection, and 44 of these patients received a three drug regimen, consisting of a 1-wk course of amoxicillin, clarithromycin, and a proton pump inhibitor (PPI)[48]. Thirty-six (82%) patients had eradication of *H. pylori* infection after completing the course of therapy. DUs healed in 49 of 54 patients after 8 wk of PPI therapy, and healed in the remaining 5 patients after 16 wk of therapy. DUs recurred within 1 year in 45% of patients remaining *H. pylori* positive despite receiving antimicrobial therapy, *vs* 48% of patients without *H. pylori* infection at enrollment. Interestingly, DU recurred within 1 year in 21 (58%) of patients with successful *H. pylori* eradication. The risk of bleeding from PUD was similar for all three groups. In a study of 28 patients with cirrhosis and PUD, including 18 with *H. pylori* infection and 10 without *H. pylori* infection, followed for up to 2 years after undergoing *H. pylori* eradication or chronic PPI therapy, *H. pylori* eradication did not prevent PUD relapse[83]. PUD relapsed in 8 of 18 *H. pylori* positive and in 9 of 10 *H. pylori* negative patients (P = 0.04). An absence of *H. pylori* infection and more severe liver disease were independently associated with shorter time to PUD relapse. In a retrospective study from Asia, 103 patients with PUD and *H. pylori,* who were eradicated late (> 1 year) after PUD diagnosis had a higher risk of recurrent PUD as compared to 154 patients with *H. pylori* eradicated early (< 1 year) (HR = 1.58, 95%CI: 1.09-2.28, P = 0.015). However, early or late eradication of *H. pylori* did not reduce the ulcer recurrence rate in patients with alcoholic cirrhosis[83,84].

NSAIDs constitute an important cause of UGI mucosal injury, including PUD, gastritis, and related complications of GI hemorrhage or GI perforation in the general population[85,86]. NSAIDs are believed to play a role in UGI bleeding from PUD in patients with advanced liver disease, although the data are relatively limited and somewhat contradictory. A recent, retrospective, nationwide, American study evaluated use of NSAIDs in 4876 cirrhotic patients hospitalized for either variceal (n = 3307) or nonvariceal (n = 1569) UGI bleeding[87]. Use of non-selective NSAIDs were associated with a nearly two-fold increased risk of both variceal and nonvariceal UGI bleeding among cirrhotic patients (OR = 1.87, 95%CI: 1.66-2.11). Also, celecoxib use was associated with a non-significant trend of a moderately increased risk of UGI bleeding (OR = 1.44, 95%CI: 0.89-2.31). Concomitant administration of PPIs or histamine-2 receptor antagonists tended to decrease UGI toxicity of non-selective NSAIDs or celecoxib.

In a prospective study, NSAID administration less than one week before hospitalization, was reported by 15 (42.8%) of 35 patients with cirrhosis and PUB, by 102 (58.2%) of 125 non-cirrhotic patients with PUB, and by 6 (8.5%) of 70 patients with cirrhosis and without PUB (P = 0.0001)[88]. This study suggests NSAIDs may play a role in the pathogenesis of PUB in cirrhotic patients.

However, in a recent, prospective study from France of 203 patients admitted to the ICU, NSAIDs or aspirin were used significantly less by cirrhotic patients with PUB [5 (17%) of 29] than by non-cirrhotic patients with PUB [94 (54%) of 174, P < 0.0001][63]. The majority of PUB in cirrhotic patients were idiopathic (*i.e.*, unrelated to *H. pylori* infection or NSAID use). Patients with advanced liver disease may avoid NSAIDs because physicians commonly warn these patients about the potential hepatotoxicity of NSAIDs[89].

DL

DL is a dilated (caliber-persistent), aberrant, submucosal, end artery that erodes through the thin overlying layer of GI mucosa in the absence of an ulcer to cause bleeding. It is responsible for approximately 1.5% of acute UGI bleeding[31]. About 70% of all DLs occur in the stomach, and about 75% of all gastric DLs occur within 6-10 cm of the gastroesophageal junction[29,90]. Although relatively uncommon, DL constitutes an important cause of acute UGI bleeding, given its propensity to cause massive, life-threatening, and recurrent bleeding[29]. Such features may be particularly clinically significant in patients with coexistent liver disease.

Data on UGI bleeding from DL in patients with underlying liver disease are limited. In a retrospective, single-center, American study of endoscopic records of 4569 consecutive patients with UGI bleeding admitted between 1991-1996, DL was the etiology of bleeding in only 6 (0.13%) patients[91]. However, 5 of these 6 patients had advanced liver disease, defined as cirrhosis or portal hypertension, including 4 patients with alcoholic liver disease. The association between UGI bleeding from DL and advanced liver disease was highly statistically significant (OR = 19.04; 95%CI: 2.1-900.8; P < 0.002), suggesting that DL may be an underappreciated cause of UGI bleeding in patients with advanced liver disease.

Other studies report a moderately strong association of UGI bleeding from DL in patients with advanced liver disease, particularly aALD. In a retrospective study of 480 patients with UGI bleeding, 28 (5.8%) had DLs[92]. Among these 28 patients with DL, 4 (14.3%) had documented alcoholic cirrhosis, and 8 others (29%) were abusing alcohol. Alcohol consumption may promote bleeding from DL because alcohol may weaken the arteriolar wall leading to vessel rupture that precipitates the bleeding[93]. Key clinical findings in DL, with an emphasis on DL associated with cirrhosis, are summarized in Table 4[29,31,90-98].

MWS

In MWS patients typically present with hematemesis from longitudinally arrayed mucosal lacerations straddling the gastroesophageal junction and extending into the distal esophagus or proximal stomach that are usually induced by antecedent forceful retching or nonbloody vomiting[99]. The mucosal lacerations cause bleeding from submucosal arteries. This lesion is reliably diagnosed by EGD. The hematemesis is typically mild. MWS accounts for approximately 5% of UGI bleeding[100]. In 40% to 80% of cases, MWS is associated with heavy alcohol consumption, either from binge drinking or chronic alcoholism, that induces the vomiting or retching[100,101]. A hiatal hernia is another important risk factor for MWS[102].

The prevalence of MWS causing UGI bleeding in patients with cirrhosis is reportedly 3%-10% which is similar to its prevalence of 5% in the general population[39,51,100,103], but MWS is likely more prevalent in patients with aALD, given its strong association with alcoholism. For example, among 339 patients with portal hypertension, confirmed by measurements of HVPG, undergoing emergency EGD for UGI bleeding, 55 patients (16.2%) had MWS[26]. Forty (73%) of these 55 patients had alcoholism as the etiology of their liver disease, which was a significantly higher rate than the rate of alcoholism in patients with other etiologies of UGI bleeding [157 (55.3%) of 284]. Moreover, the frequency of bleeding from MWS was significantly higher in patients with more advanced cirrhosis (Child-Pugh class B or C).

In a study of 42 patients with bleeding from MWS, 14 (33%) had underlying liver disease, including six (14%) with aALD[27]. Patients with liver disease had significantly more severe MWS bleeding than

Table 4 Key clinical findings in Dieulafoy's Lesion associated with chronic liver disease

Key findings	Rationale	Ref.
Typically presents with acute severe bleeding	Micropulsatile bleeding produced by rent in an arteriole which is under high pressure	Nojkov *et al*[31], Luis *et al*[94]
Bleeding typically painless	Primary vascular event (bursting of a persistent-caliber vessel) without associated inflammation or ulceration	Cappell[29]
Appears at endoscopy as an elevated pigmented protuberance with minimal surrounding erosion and no ulceration	Formed by a caliber-persistent artery that erupts through superficial overlying cells on mucosal surface	Nojkov *et al*[31], Lee *et al*[93]
Lesion most commonly located in stomach, typically within 6 cm below the gastroesophageal junction along the lesser curve	This gastric region is not perfused by a submucosal plexus, but instead is perfused directly from tributaries of the right and left gastric arteries	Cappell *et al*[29], Fockens *et al*[90], Lee *et al*[95]
Often (up to 30% of cases) missed at initial esophagogastroduodenoscopy (EGD)	Missed at EGD because lesion is small and inconspicuous	Nojkov *et al*[31], Chung *et al*[96]
Incidence of 1.5% among general population of patients with upper GI bleeding		Fockens *et al*[90], Chaer *et al*[97]
High (25%) mortality if untreated at EGD, which is reduced to about 10% with endoscopic therapy	High risk of rebleeding if not treated endoscopically. Rebleeding is frequently massive	Romãozinho *et al*[98]
Dieulafoy's lesion may be associated with cirrhosis		Akhras *et al*[91], Baettig *et al*[92]
Bleeding from a Dieulafoy's lesion is associated with alcoholism	Alcohol may precipitate DL rupture manifesting as GI bleeding by weakening the dilated (caliber-persistent) arteriolar wall in Dieulafoy's lesion	Baettig *et al*[92], Lee *et al*[95]

GI: Gastrointestinal; EGD: Esophagogastroduodenoscopy.

patients without liver disease as indicated by units of packed erythrocytes transfused ($P < 0.005$), but MWS bleeding severity was not correlated with the presence of portal hypertension or Child-Pugh score. Only three patients (21%) required endoscopic therapy; in these three patients epinephrine injection and/or Bicap electrocoagulation successfully stopped the bleeding or rebleeding[27]. There were no fatalities from MWS bleeding in patients with liver disease. Contrariwise, in a small trial of 30 patients with UGI bleeding from MWS, the bleeding was significantly more severe and more difficult to control at EGD in the 8 patients with portal hypertension than in the 22 patients without portal hypertension[28]. Additionally, patients with portal hypertension were more likely to rebleed from MWS [3 (38%) of 8 patients rebled] as compared to patients without portal hypertension [0 (0%) of 22 patients rebled, $P = 0.02$, Fisher's exact test, P value calculated by us from original descriptive statistics]. All 8 patients with portal hypertension and MWS bleeding had underlying aALD[28]. A Korean study of 159 patients with acute UGI bleeding from MWS also reported a higher frequency of recurrent bleeding from MWS in patients with cirrhosis[82]. Cirrhosis was present in 33 (21%) of these patients. Seven (41%) of the 17 patients who rebleed from MWS had cirrhosis ($P = 0.02$). The vast majority of patients who rebled were alcoholics [14 (82%) of 17 patients].

Another study of 224 patients, recruited over 10 years, with acute UGI bleeding from MWS revealed that 68 (31%) of the patients had abused alcohol and had aALD[42]. The bleeding stopped spontaneously in 90% of the 224 patients, with only 20 patients requiring endoscopic or surgical therapy. However, 8 of the patients died, including six patients who died from the acute UGI bleeding from massive hemorrhage, pulmonary aspiration, or hemorrhagic shock, and two patients who died from hepatic complications indirectly related to the acute UGI bleeding.

The prevalence of acute UGI bleeding from MWS in patients with liver disease may be lower in Asian than Western countries. In a recent, prospective, single-center, study from China, 16 (3%) of 519 patients with acute nonvariceal UGI bleeding had MWS. Only one of these patients had cirrhosis[104]. Key clinical features of acute UGI bleeding from MWS in patients with underlying liver disease are summarized in Table 5[27,28,38,82,100,101,105-107].

Treatment

Pre-endoscopic therapy: Most patients with severe liver disease should be admitted to an intensive care unit (ICU) for acute UGI bleeding, particularly when manifesting clinical findings suggestive of severe UGI bleeding, including unstable vital signs, active hematemesis, or hematochezia, or when patients have severe comorbidities. Clinical stratification of variceal *vs* nonvariceal etiology for UGI bleeding is difficult before EGD in these patients because some patients with stigmata of underlying chronic liver disease bleed from PUD, DL, or MWS, as aforementioned. However, physical findings of portal hypertension, such as ascites or caput medusa, favor the diagnosis of variceal bleeding[108]. Endotracheal intubation, to protect the airway, should be considered in patients with severe hematemesis, suspected variceal bleeding, or significant hepatic encephalopathy[109,110]. A Foley catheter is inserted in patients with shock, unstable vital signs, oliguria, or massive bleeding. Nasogastric lavage is often helpful to assess whether the bleeding

Table 5 Summary of clinical features of acute gastrointestinal bleeding from Mallory-Weiss syndrome in patients with underlying liver disease

Key findings	Ref.
Findings generally associated with Mallory-Weiss syndrome	
Characterized by longitudinally oriented mucosal lacerations in the distal esophagus or very proximal stomach	Knauer[101]
Accounts for about 5% of acute upper GI bleeding	Michel *et al*[100]
Mortality of bleeding from Mallory-Weiss syndrome is only about 3%-5%. Risk factors for mortality include age > 65 yr and significant comorbidities	Ljubičić *et al*[105]
Findings associated with Mallory-Weiss syndrome associated with alcoholism	
MWS strongly associated with alcoholism	Knauer[101]
MWS very frequently (40%-80%) associated with alcoholism or recent binge drinking	Watts *et al*[106]
Overall prevalence of bleeding from MWS in cirrhosis is up to 10%	del Olmo *et al*[38], Feng *et al*[82]
Alcoholics with portal hypertension have higher prevalence of bleeding from MWS (up to 16%)	Paquet *et al*[26], Schuman *et al*[27]
MWS may be the first bleeding episode in > 1/3 (37%) of these patients	
Patients with cirrhosis have more severe bleeding from MWS and more likely to rebleed from MWS (compared to non-cirrhotics)	Schuman *et al*[27], Jensen *et al*[28], Kim *et al*[107]
Re-bleeding risk particularly high in alcoholics	
Contradictory data on effect of portal hypertension on severity of bleeding from MWS	Schuman *et al*[27], Jensen *et al*[28]
Bleeding from MWS can precipitate liver failure with its attendant mortality in about 3% of patients with alcoholic cirrhosis	del Olmo *et al*[38]

GI: Gastrointestinal; MWS: Mallory-Weiss syndrome.

is from the UGI tract, to assess the tempo of the bleeding, and to clear the gastric field for EGD. This is most helpful in the presence of severe, active, bleeding often associated with variceal or other UGI bleeding in cirrhotic patients.

As for any acute UGI bleeder, volume resuscitation is critical in patients with cirrhosis to prevent systemic hypotension and consequent end-organ damage to heart, brain, or kidneys from hypoperfusion. At least two, large-caliber, peripheral intravenous lines, or a reliable central venous line should be inserted. Volume resuscitation is initially accomplished by crystalline solution, using normal saline or Ringer's lactate, but transfusion of packed erythrocytes is often required, after typing and crossing of blood. The number of units transfused is guided by the tempo of the UGI bleeding, the vital signs, and serial hematocrit determinations. It is important, however, to avoid over-transfusion in patients with underlying advanced liver disease, as this can precipitate variceal bleeding by increasing the portal hypertension and refilling of the varices[13,111]. Packed erythrocytes should be transfused to maintain the hemoglobin level only at about 8 g/dL. However, patients who have cardiovascular disease and severe comorbidities, or are extremely elderly should be transfused to a somewhat higher level[112].

Coagulopathies are appropriately treated. Thrombocytopenia is common in patients with advanced liver disease because of splenic sequestration from splenomegaly and direct bone marrow toxicity of alcohol[113]. Furthermore, platelet counts decline directly from platelet loss during acute UGI bleeding. Platelets should be transfused to maintain > 50000 platelets/mm^3 in actively bleeding patients[114]. Coagulopathy from liver disease should by monitored by the INR, and should be corrected in actively bleeding patients, usually by administration of fresh frozen plasma (FFP). One unit of FFP should also be transfused after transfusion of every four units of packed erythrocytes to replenish depleted clotting factors[115].

Electrolyte abnormalities are appropriately treated. Patients transfused large volumes of blood products should be carefully monitored and treated for hypocalcemia because citrate present in blood products binds to ionized calcium[116]. These patients have an increased risk of hypophosphatemia and hypokalemia, especially if dextrose solutions are used during resuscitation, because insulin release consequent to dextrose administration accelerates the transfer of phosphate and potassium into cells[117]. Prophylactic antibiotics should be administered at presentation, because antibiotics improve survival in cirrhotic patients with UGI bleeding, especially with bleeding from esophageal varices[118], by reducing the risk of spontaneous bacterial peritonitis or other severe infections[119].

Alcoholics should receive thiamine. They should be monitored for symptoms and signs of alcohol withdrawal. Alcohol withdrawal may be difficult to appreciate in alcoholics suffering from variceal bleeding because tachycardia, confusion, and low-grade pyrexia may be attributed to the variceal bleeding alone. Special considerations in the therapy of acute UGI bleeding in patients with aALD are summarized in Table 6[25,109,110,117-129].

All patients presenting with acute UGI bleeding receive PPI therapy intravenously before endoscopy even though many patients are bleeding from lesions that are not acid-mediated, because the neutralization of intraluminal gastric acid with PPI therapy stabilizes blood clots for all gastric lesions[130,131]. For example, PPIs are standardly administered before EGD even in cirrhotic patients with suspected variceal bleeding. Patients with suspected esophageal variceal bleeding or other bleeding related to portal hypertension, based

Table 6 Special considerations in therapy for alcoholics with liver disease presenting with acute upper gastrointestinal bleeding

Recommended clinical practice	Rationale	Ref.
Consider early intubation for severe upper GI bleeding in a patient with alcoholism or alcoholic cirrhosis	These patients are at higher risk of aspiration because variceal bleeding related to alcoholism or cirrhosis is frequently massive, arises from the esophagus which is much closer to the trachea than other types of gastroduodenal bleeding; and the patient may be obtunded from hepatic encephalopathy from cirrhosis	Herrera[109], Rudolph *et al*[110]
Avoid sedatives and narcotics in patients with advanced liver disease	May precipitate hepatic encephalopathy from cirrhosis	Bamji *et al*[120], Prabhakar *et al*[121]
Monitor for hepatic encephalopathy	Patients with advanced cirrhosis at risk for hepatic encephalopathy	Rahimi *et al*[122]
Monitor for delirium tremens	Acute alcoholic withdrawal in hospital can induce delirium tremens	Ferguson *et al*[123], Holloway *et al*[124]
Avoid over-transfusion (maintain hemoglobin level at about 8 gm/dL)	Over-transfusion may exacerbate variceal bleeding by increasing portal hypertension	Herrera[109]
Patients often have thrombocytopenia which may contribute to the bleeding	Thrombocytopenia due to splenic sequestration from splenomegaly from portal hypertension and from direct alcohol toxicity to bone marrow	Pradella *et al*[125]
Patients often have a prolonged INR which may contribute to the bleeding	INR prolonged due to inadequate synthesis of liver-dependent clotting factors, such as factor V, due to advanced liver disease	Lata *et al*[126]
Administer thiamine	Prevent Wernicke's syndrome from thiamine deficiency which is common in alcoholics	Hack *et al*[127]
Monitor for electrolyte abnormalities which may be more prominent in alcoholics		Knochel[117]
Consider early (urgent) esophagogastroduodenoscopy	Important to distinguish esophageal variceal bleeding from other etiologies of upper GI bleeding because esophageal variceal bleeding has different therapies	Buccino *et al*[37], del Olmo *et al*[38]
Consider empiric octreotide therapy before endoscopy	Alcoholics or patients with cirrhosis frequently have GI bleeding from esophageal varices which can be treated by octreotide therapy	Ludwig *et al*[128]
Perform paracentesis, as necessary, to exclude spontaneous bacterial peritonitis	Patients with cirrhosis and ascites are at high risk to develop spontaneous bacterial peritonitis due to mild immunosuppression with cirrhosis	Goulis *et al*[119]
Administer antibiotics in the presence of acute GI bleeding in a cirrhotic patient	Empiric antibiotic therapy lowers mortality because of decreased sepsis	Bernard *et al*[118]
Monitor BUN and creatinine levels to detect early hepatorenal syndrome. Avoid nephrotoxic medications such as NSAIDs	At high risk for renal deterioration due to decreased renal perfusion associated with cirrhosis and hypovolemia from GI hemorrhage	Ginès *et al*[129]
Exclude acute portal vein thrombosis in patients who suddenly develop severe esophageal varices by abdominal imaging studies (*e.g.*, Doppler ultrasound or CT angiography)	Portal vein thrombosis in a patient with preexistent cirrhosis may exacerbate the portal hypertension and cause acute variceal bleeding	D'Amico *et al*[25]

GI: Gastrointestinal; INR: International normalized ratio; BUN: Blood urea nitrogen; NSAIDs: Nonsteroidal anti-inflammatory drugs.

on patient history, stigmata of chronic liver disease, or abnormal liver function tests, are generally treated with octreotide before EGD to decrease splanchnic blood flow and reduce the risk of bleeding from potential esophageal varices[132].

Diagnostic endoscopy: All patients with acute UGI bleeding generally require EGD, but patients with aALD or nonalcoholic cirrhosis generally require more urgent EGD because of the potential for life-threatening variceal bleeding requiring endoscopic therapy. EGD is highly sensitive and specific for diagnosing the etiology of acute UGI bleeding, with a diagnostic sensitivity of about 95%[133]. UGI bleeding from esophageal varices, portal hypertensive gastropathy, and GAVE are reliably diagnosed at EGD, as are bleeding from PUD or MWS. Occasionally, PUD or a Mallory-Weiss tear may be obscured by active bleeding, or a peptic ulcer may be obscured by an overlying pool of blood or adherent clot. Contrariwise, DL is missed in up to 30% of initial EGDs, especially if the patient is not actively bleeding or oozing at EGD, because of its small size and inconspicuousness and patients sometimes require repeat EGD for the diagnosis[134].

General principles of endoscopic therapy: Focal GI lesions, including PUD, DL, or MWS, are treated by focal endoscopic therapies, including injection, ablation, and mechanical therapy (Table 7)[31,135]. Injection therapy most commonly involves local injection of epinephrine or sclerosing agents (sclerotherapy). Epinephrine injection induces vasospasm and tamponade/mechanical pressure from interstitial injection which promotes stasis, thrombosis, and hemostasis[136]. It is generally diluted in saline to a concentration of 1:10000 and injected in four quadrants circumferentially around a point lesion, such as a peptic ulcer. Relative contraindications to epinephrine therapy include severe tachycardia, life-threatening cardiac arrhythmias such as atrial flutter, unstable

Table 7 Local endoscopic therapies

Injection therapies
Dilute epinephrine
Sclerotherapy
Ablation therapies
Contact methods
Thermocoagulation: heater probe
Electrocoagulation: BICAP (bipolar electrocoagulation probe), Gold Probe
Noncontact methods
APC (argon plasma coagulation)
Mechanical therapy
Banding
Hemoclips
Endoscopic suturing

vital signs from untreated, profound, hypovolemia, and recent myocardial infarction or unstable angina[31]. However, cardiovascular side effects, including angina, tachycardia, cardiac arrhythmias and systemic hypertension, are relatively uncommon[137]. Epinephrine injection monotherapy arrests about 80% of active bleeding[135]. For example, in a prospective study, 66 (85%) of 78 patients undergoing endoscopic epinephrine injection for a nonbleeding visible vessel or actively bleeding ulcer had no recurrent bleeding[138]. Sclerotherapy promotes vascular inflammation and thrombosis from local tissue irritation. Cyanoacrylate, thrombin, or fibrin glue are infrequently used as injection therapies[139]. Cyanoacrylate hardens as a glue to plug a bleeding artery[140].

Ablation modalities include thermocoagulation, electrocoagulation, and argon plasma coagulation (APC) (Table 7). These modalities arrest bleeding by focally applying intense energy, *via* heat, electricity or a plasma cloud, to destroy and devitalize tissue. In APC the probe is hovered over the lesion without lesion contact[141]. Contrariwise, thermocoagulation and electrocoagulation require direct contact (apposition) of the probe to the lesion, and application of pressure during coagulation (coaptive coagulation) to compress and seal any bleeding vessel[31,135,142]. Nd:YAG (neodymium-doped yttrium aluminum garnet) laser photocoagulation has become obsolete because it can cause deep injury and has an unacceptably high risk of gastrointestinal perforation of approximately 3%[143].

Mechanical therapies, including band ligation, hemoclips, or over the scope endoclips (bear claw), arrest bleeding by mechanically occluding a bleeding vessel[144]. Mechanical therapy requires greater endoscopic skill and experience than injection or ablative therapies because the band or clip must be placed precisely around the bleeding lesion to successfully strangulate it[135]. Mechanical therapy appears to be the therapy of choice in the presence of a moderate-to-severe coagulopathy because it mechanically occludes a bleeding vessel without depending upon thrombosis to stop the bleeding[135]. Thus mechanical therapy may be favored in cirrhotic patients with severe thrombocytopenia or a highly elevated international normalized ratio (INR). Table 7 lists common endoscopic therapies for nonvariceal upper GI bleeding.

Endoscopic therapy for PUD: In a patient with recent bleeding from PUD, therapeutic EGD is generally performed according to endoscopic SRH (Table 8; Forrest classification)[145]. Patients with major SRH at EGD, including active bleeding, oozing, or a nonbleeding visible vessel, should undergo endoscopic therapy[135]. A nonbleeding visible vessel is defined endoscopically as an elevation (projection) from the ulcer base that is pigmented, whether it be red, purple, blue, or gray[146]. The rationale for endoscopic therapy is to prevent rebleeding or ongoing bleeding from PUD, because patients with rebleeding have a very high mortality of up to 30%[147]. For example, patients with a nonbleeding visible vessel have a 40%-60% risk of rebleeding with medical therapy alone that is reduced to about 15% with dual endoscopic therapy[148]. Patients with minor endoscopic SRH (Table 8) should not undergo endoscopic therapy because of a low risk of rebleeding and mortality even without endoscopic therapy. For example, patients with an ulcer that is homogeneous and clean-based or that has a flat pigmented spot have an about 3%-5% or 7%-10% risk, respectively, of rebleeding, even without undergoing endoscopic therapy[147].

Endoscopic therapy for a nonbleeding, adherent clot is somewhat controversial. Many endoscopists, however, advocate aggressive removal of adherent clots. First, the endoscopic diagnosis is often ambiguous without clot removal. A presumed benign ulcer underneath an adherent clot may be exposed as a malignant ulcer or DL after clot removal[135]. Second, clot removal may reveal unexpected underlying major SRH, such as a nonbleeding visible vessel, that mandates endoscopic therapy. Third, even though mechanical clot removal occasionally precipitates active bleeding, endoscopists generally prefer to treat active bleeding immediately at the initial EGD rather than passively wait for potential rebleeding that would necessitate repeat EGD. At EGD, an adherent clot is initially vigorously irrigated using a water pump. If irrigation fails to remove the clot, the clot is decapitated using a snare without electrocoagulation after four-quadrant injection around the clot with epinephrine[149]. Endoscopic therapy is applied to the exposed underlying ulcer, especially if active bleeding or oozing occurs after clot removal or a visible vessel is exposed[150]. Multiple studies support forceful removal of adherent clots. For example, in a randomized, controlled trial of 32 patients with UGI bleeding and nonbleeding adherent clots identified at EGD, patients undergoing endoscopic epinephrine injection and mechanical clot removal followed by thermocoagulation had a 5% rebleeding rate, whereas those receiving medical therapy with a PPI had a 34% rebleeding rate[151]. An adherent clot should generally

Table 8 Classification of endoscopic stigmata of recent hemorrhage for acute upper gastrointestinal bleeding from peptic ulcer disease

Endoscopic SRH	Endoscopic appearance	Endoscopic therapy	Endoscopic therapy and rationale for therapy
Major SRH			
Active bleeding	Active bleeding observed at EGD	Yes	Reduction from 90% to 15% risk of ongoing bleeding with performance of endoscopic therapy
Nonbleeding visible vessel	Pigmented elevation (projection) from ulcer base, whether red, blue or gray in color	Yes	Reduction from about 50% to 15% risk of rebleeding with performance of endoscopic therapy
Intermediate SRH			
Adherent clot	Focal clot that is resistant to removal by mild-to-moderate irrigation	Recommended by most endoscopists	
Active oozing of blood	Active oozing observed at EGD	Generally recommended	May reduce risk of rebleeding from 28% to 15% with endoscopic therapy
Minor SRH			
Flat pigmented spot	Pigmented spot, whether red, blue or gray, which lies flat on the ulcer base	No	Low risk of rebleeding of about 13% with medical therapy alone
No SRH			
Homogeneous, clean-based ulcer	Simple ulcer with no bleeding, no adherent clot, no visible vessel and no pigmented spot	No	Extremely low risk of rebleeding of about 4% that does not warrant the risks of endoscopic therapy

Adapted from Cappell[135]. SRH: Stigmata of recent hemorrhage; EGD: Esophagogastroduodenoscopy.

be considered for forceful removal if the lesion is at a location amenable to endoscopic therapy and if the endoscopist is comfortable with the techniques of forceful endoscopic clot removal and therapy.

Epinephrine injection is the most common initial endoscopic therapy for PUD with high or intermediate SRH because this therapy is relatively easy and quick to apply, even in the face of severely active bleeding, and can help clear the endoscopic field by reducing the tempo of active bleeding to enable application of more efficacious, pinpoint, therapy. Epinephrine therapy, by itself, is not highly efficacious at achieving permanent hemostasis, with a rebleeding rate of about 20%[152]. Endoscopists, therefore, generally perform a second endoscopic therapy after epinephrine injection to further reduce the bleeding rate to 10%-15%[152,153]. Second therapies include ablation with thermocoagulation or electrocoagulation, and mechanical therapy with hemoclips or banding. The choice of second technique depends upon equipment availability and the training and experience of the endoscopist.

Currently employed endoscopic therapies generally have a small, acceptable risk of gastrointestinal perforation, local tissue necrosis, or bleeding exacerbation[154]. For example, the risk of perforation for all current endoscopic therapies is 1% or less[155].

A comprehensive review of the literature revealed no large studies focused on endoscopic therapy for PUD in patients with cirrhosis or aALD. However, endoscopic therapy in patients with major SRH is apparently more important in this patient population than in the general population because of the higher risks of rebleeding or dying from PUB in this population. Currently the same endoscopic therapies should be performed in this population as in the general population. It is important to treat coagulopathy from thrombocytopenia or an elevated INR for endoscopic therapy to be most effective.

Endoscopic therapy for DL: Endoscopic therapy is uniformly performed for DLs, even if SRH are absent, because of the high risk of rebleeding from DLs without endoscopic therapy and the high mortality of this rebleeding. Reducing this high risk of rebleeding justifies the risks of endoscopic therapy[31]. Endoscopic therapy reduces the risk of rebleeding from DLs from 30% to about 10%[156].

The data on individual endoscopic therapies for DL are limited because DL is relatively uncommon. Many endoscopic therapies are generally effective for DLs in the general population. Current data suggest that mechanical hemostasis may be the most effective. A recent, large, literature review encompassing 106 patients undergoing hemoclips and 86 patients undergoing band ligation as monotherapies for bleeding DLs showed that both techniques were nearly always effective at achieving initial hemostasis, and had a low (< 10%) rate of rebleeding[31].

In the same recent literature review, among 68 patients undergoing epinephrine injection therapy and among 13 patients undergoing sclerotherapy, about 12% failed primary hemostasis, and another 25% rebleed after initial hemostasis[31]. Although this efficacy is somewhat lower than that for mechanical therapy, injection therapy appears to be an excellent initial therapy for massive bleeding from DL due to ease and rapidity of the injection so that the DL can then be well visualized to apply mechanical or ablative therapy[31].

Data on efficacy of endoscopic ablation therapies for DL are somewhat limited, with only about 40 reported cases among the 3 different therapies of thermocoagulation, electrocoagulation, and APC, reported in 7 small clinical studies[31]. However, despite these limitations, the data demonstrate that endoscopic ablation is relatively efficacious, with a combined initial

Table 9 Efficacy of endoscopic therapy for gastrointestinal bleeding from Dieulafoy's lesion in cirrhotics

Endoscopic procedure (Number of patients)	Hemostatic technique	Lesion location	Study type	Follow-up	Patient outcome	Ref.
EGD (12)[1]	Epinephrine + Polidocanol	Stomach/ duodenum	Retrospective	5 mo	No rebleeding, 3 of cirrhotic pts died from hepatic decompensation within 5 mo	Baettig *et al*[92]
EGD (6)	Not reported	Stomach/ duodenum	Retrospective	NA (6 yr period reviewed)	No rebleeding in 5 of 6 pts (83%), 1 pt (17%) underwent surgery for rebleeding	Akhras *et al*[91]
EGD (5)	Histoacryl (3) Epinephrine + heater probe (2)	Stomach	Retrospective	18 mo	No rebleeding	Cheng *et al*[159]
EGD (4)	Hemoclip	Stomach/ duodenum	Prospective	54 mo	9% of whole cohort of 34 pts rebled. No data whether any of the cirrhotics rebled	Yamaguchi *et al*[160]
EGD (2)	Rubber band ligation	Stomach	Retrospective	30 d	No rebleeding, 1 of 2 pts died from hepatic decompensation within 30 d	Mumtaz *et al*[157]
EGD (1)	Argon plasma coagulation	Stomach	Retrospective	29 mo	No rebleeding	Iacopini *et al*[158]

[1]Includes 4 patients with cirrhosis and 8 patients who actively abused alcohol. EGD: Esophagogastroduodenoscopy; pts: Patients.

success rate of hemostasis of about 95%-97.5% and a rebleeding rate of 12.5%[31].

In summary, the data show that endoscopic therapy is highly effective at achieving initial hemostasis and preventing rebleeding from DLs. Mechanical therapy may be the most effective endoscopic therapy. However, dual endoscopic therapy, with initial epinephrine injection followed by mechanical therapy, may be even more effective than any monotherapy. The high risk of rebleeding from DL justifies the risk of undertaking endoscopic therapy to prevent further bleeding[31].

Limited data exist on the effectiveness of endoscopic therapy for DL in cirrhotic patients. Table 9 summarizes data extrapolated from multiple studies on the efficacy of various endoscopic techniques[91,92,157-160]. Overall, various endoscopic hemostatic techniques, including injection, ablation, and mechanical therapy, can be effectively applied to bleeding DLs in cirrhotic patients. However, patients with cirrhosis likely have increased mortality related to hepatic decompensation. For example, four (18%) of the 22 reported patients in Table 9 died within 5 mo of the UGI bleeding, from hepatic decompensation, despite achieving successful endoscopic hemostasis.

In one study of 28 patients with DL, including 4 with documented cirrhosis and 8 alcoholics, endoscopic therapy with epinephrine and polidocanol injection successfully stopped the bleeding in 96% of cases[92]. The bleeding was effectively controlled in 4 patients with alcoholic cirrhosis, without rebleeding or death during the initial hospitalization, but 3 of the 4 cirrhotic patients died during a mean follow-up period of 5 mo, including 2 dying from hepatic coma and 1 dying from variceal bleeding. In a retrospective study of 23 patients with DL-induced acute UGI bleeding, including 2 (8.7%) patients with advanced liver disease, there was no difference in short-term (30 d) clinical outcomes between patients treated with endoscopic rubber band ligation *vs* endoscopic thermal/injection therapy[157]. Six patients (26%) died within 30 d after bleeding from DL, including 1 of the 2 patients with advanced liver disease, who expired from liver decompensation. In an Italian study with long-term mean follow-up of 29 mo, APC was a safe and effective treatment for 23 patients with GI bleeding from DL including 1 patient (4.3%) with liver cirrhosis[158]. None of the patients died and only 1 patient experienced rebleeding, which was successfully retreated with APC.

Endoscopic therapy for MWS: Endoscopic therapy for MWS depends upon the presence of SRH and other factors. Endoscopic therapy should be applied for MW tears that are actively bleeding at endoscopy. Endoscopic therapy may be considered when other SRH are present, including a protruding visible vessel, pigmented protuberance, or fresh adherent clot, especially in the presence of risk factors for rebleeding of initially severe bleeding or a coagulopathy. However, most patients with MWS do not require endoscopic therapy because the tears usually heal spontaneously with only medical therapy[161]. In particular, endoscopic therapy is usually unnecessary when the bleeding is minor as indicated by absence of hematochezia, stable vital signs at presentation, and lack of blood transfusions; when a coagulopathy is not present; and when SRH are absent.

Data on endoscopic therapies for MWS in the general population are somewhat limited by the retrospective nature of most studies and the relatively small number of patients in individual studies. Currently recommended endoscopic therapies include injection therapy with epinephrine, ablation therapy with electrocoagulation, and mechanical therapy with hemoclips or band ligation. Epinephrine can be used as a monotherapy or combined with a second therapy. For example, in a randomized, controlled trial of bleeding from high-risk MWS, bleeding

Table 10 Efficacy of endoscopic therapy for gastrointestinal bleeding from Mallory-Weiss syndrome in cirrhotics

Number of patients	Hemostasis Technique	*n* (%) with active alcohol abuse	Time of EGD	Study type	Follow-up	Outcome	Ref.
55	Aetoxysklerol injection	40 (73)	Within 6 h	Retrospective	2 yr	No rebleeding or death	Paquet *et al*[26]
14[1]	Epinephrine injection/electrocoagulation	6 (43)	Within 24 h	Retrospective	5 yr	No rebleeding or death[2]	Schuman *et al*[27]
7	Band ligation 4 Hemoclips and epinephrine 3	Not reported	Within 12 h	Retrospective	5 d	No rebleeding or death	Lecleire *et al*[165]
3	Heater probe/Bicap electrocoagulation	3 (100)	"Urgently" after presentation	Retrospective	17 mo	Failed to control bleeding in 1/3 pts (33%)	Jensen *et al*[28]
1	Hemoclip and Endoscopic band ligation	1 (100)	"Emergently" at presentation	Retrospective	NA	Failed to stop bleeding with both techniques; patient underwent surgery and died of liver failure 3 d after surgery	Yin *et al*[104]

[1]Only 3 of the 14 patients treated endoscopically; [2]Three deaths reported out of the overall cohort of 42 patients presenting with bleeding from MWS. None of the deaths were related to MWS or liver disease. EGD: Esophagogastroduodenoscopy; MWS: Mallory-Weiss syndrome.

recurred in 6.8% of patients receiving endoscopic injection therapy *vs* 25.8% of patients receiving medical therapy alone ($P < 0.05$)[162]. Electrocoagulation significantly reduced the risk of rebleeding compared to medical therapy in patients with MWS at high risk of rebleeding[163]. Likewise, endoscopic band ligation and hemoclip placement were highly effective at preventing rebleeding from MWS, as demonstrated in a randomized clinical trial of 41 patients[164].

Table 10 summarizes the data on the efficacy of endoscopic therapies to treat acute MWS bleeding in cirrhotic patients[26-28,104,165]. Although the data are limited and retrospective, all the reported therapeutic endoscopic modalities appeared to be safe and effective to control the bleeding in the vast majority of cirrhotic patients. In the aforementioned study by Paquet *et al*[26] which included 339 consecutive patients with cirrhosis and portal hypertension who underwent EGD for acute UGI bleeding within 6 h of presentation, 55 patients had bled from MWS. All 55 patients were treated endoscopically with injection of aethoxysklerol into the bleeding lesion, which universally resulted in successful hemostasis, with no rebleeding or other adverse effects[26]. In another aforementioned study, 14 patients with cirrhosis and acute bleeding from MWS underwent EGD within 24 h of presentation, but endoscopic therapy (epinephrine injection or electrocoagulation) was deemed necessary in only 3 (21%) of the patients[27]. No patient experienced rebleeding, other complications, or death related to the MWS or the underlying liver disease. Mechanical endoscopic hemostasis (band ligation or hemoclip placement) was also reported effective in cirrhotic patients with bleeding MWS. In a recent French study of 56 patients comparing these 2 mechanical therapies, none of the 7 patients with cirrhosis, treated with band ligation in 4 and hemoclips placement and epinephrine in 3, rebleed or suffered endoscopic complications. On multivariate analyses, liver disease was not a risk factor for rebleeding.

However, endoscopic therapy is not universally successful in patients with cirrhosis who bleed from MWS. For example, a small report of 8 patients with cirrhosis and MWS bleeding, reported endoscopic failure to control the initial MW bleeding in 1 (33%) of the 3 patients receiving either heater probe thermocoagulation or Bicap electrocoagulation. Additionally, a recent case-series of bleeding MWS from China included one patient with concomitant alcoholic liver cirrhosis. This patient was the only one in this study who failed endoscopic therapy initially with hemoclips placement and subsequently hemoclips and band ligation and died 3 d after undergoing surgery from liver failure[104].

DISCUSSION

The incidence of PUD, frequency of bleeding from PUD, and severity of bleeding from PUD are all increased in patients with cirrhosis, including those with aALD. However, the pathophysiology of this phenomenon is incompletely understood. Experiments in laboratory animals, such as rats, may help elucidate the pathophysiology, by separately analyzing the effects of alcohol exposure and portal hypertension (*e.g.*, expose rats to alcohol consumption *vs* experimentally ligating or banding the portal vein to produce experimental portal hypertension with little acute hepatocellular liver injury).

The clinical presentation of PUD associated with cirrhosis or aALD is incompletely understood because many of the studies on the subject lump PUD in cirrhotics with the other etiologies of UGI bleeding not due to portal hypertension (Table 2). Studies are needed which systematically compare patients

with PUD and cirrhosis or aALD to patients with PUD without cirrhosis or aALD. The data on endoscopic therapies for MWS and DL for patients with cirrhosis are limited. However, conclusions can be extrapolated from data on endoscopic therapies in noncirrhotic patients bleeding from these lesions. Analysis of larger patient populations of patients bleeding from MWS or DL would be useful to show that the general principles of endoscopic therapy pertain to cirrhotic patients.

H. pylori appears to play only a minor role in the increased observed rate of PUD in cirrhotics. Perhaps the most convincing evidence of this minor role is that *H. pylori* eradication does not protect against DU recurrence, as it does in the general population.

In conclusion, this review describes more frequent and more severe GI bleeding from PUD, DL, or MWS in patients with aALD than in the general population. These differences have important consequences in the clinical management and prognosis of these conditions in patients with aALD. It is hoped that this review will stimulate further study of this clinically important but incompletely understood subject.

COMMENTS

Background

Excessive alcohol consumption is highly prevalent worldwide and can cause several forms of liver injury ranging from alcoholic fatty liver disease to alcoholic hepatitis to alcoholic cirrhosis. While the majority of acute gastrointestinal bleeding with advanced alcoholic liver disease (aALD), including alcoholic hepatitis or alcoholic cirrhosis, is related to portal hypertension, about 30%-40% of acute gastrointestinal (GI) bleeding in patients with aALD is unrelated to portal hypertension. This work shows that patients with aALD have more frequent and more severe bleeding from the following etiologies of nonvariceal upper GI bleeding: peptic ulcer disease (PUD), Dieulafoy's lesion (DL), and Mallory-Weiss syndrome (MWS). Clinicians should appreciate the important clinical implications of these phenomena.

Research frontiers

It is important for clinical practitioners and clinical researchers to appreciate that patients with advanced alcoholic liver disease, including alcoholic hepatitis and alcoholic cirrhosis, have more frequent and more severe nonvariceal upper GI bleeding from PUD, DL, and MWS. This work systematically reviews and critically analyses the literature on this quickly evolving subject and reports the clinical consequences of these findings.

Innovations and breakthroughs

This review is distinguished by being a systematic review of the subject of nonvariceal bleeding in patients with aALD, by reviewing all three etiologies of non-variceal upper GI bleeding severely affected by aALD, and by a focus on the clinical implications of the reported findings. This work aims to provide practicing clinicians and clinical researches an in-depth and up-to-date comprehensive and critical review of this clinically important, but underappreciated, subject.

Applications

The currently reported increased frequency and increased severity of bleeding from PUD, DL, and MWS in patients with aALD have clinically important implications regarding the treatment, natural history, and prognosis of these etiologies of upper GI bleeding in patients with aALD. This work also stimulates clinical researchers to better understand the pathophysiology of these reported clinical phenomena.

Terminology

aALD refers to alcoholic hepatitis and alcoholic cirrhosis. This term is presently used because both of these forms of alcoholic liver disease affect the pathophysiology, natural history, treatment, and prognosis of non-variceal upper GI bleeding from PUD, DL, and MWS.

Peer-review

The manuscript provides a comprehensive review of the recent literature data on the association between PUD, DL, and MWS and aALD or cirrhosis. It is also well supported by the quoted references. This is a well written and interesting contribution of considerable interest to the readership of the Journal.

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