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World Journal of Gastroenterology

Contents

Weekly Volume 21 Number 42 November 14, 2015

EDITORIAL

TOPIC HIGHLIGHT

Contents

World Journal of Gastroenterology
Volume 21 Number 42 November 14, 2015

MINIREVIEWS

META-ANALYSIS

Contents

World Journal of Gastroenterology
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EDITORIAL

Hepatic immune tolerance induced by hepatic stellate cells

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Abstract

The liver, which is a metabolic organ, plays a pivotal role in tolerance induction. Hepatic stellate cells (HpSCs), which are unique non-parenchymal cells, exert potent immunoregulatory activity during cotransplantation with allogeneic islets effectively protecting the islet allografts from rejection. Multiple mechanisms participate in the immune tolerance induced by HpSCs, including the marked expansion of myeloid-derived suppressor cells (MDSCs), attenuation of effector T cell functions and augmentation of regulatory T cells. HpSC conditioned MDSC-based immunotherapy has been conducted in mice with autoimmune disease and the results show that this technique may be promising. This article demonstrates how HpSCs orchestrate both innate immunity and adaptive immunity to build a negative network that leads to immune tolerance.

Key words: Hepatic stellate cells; Myeloid-derived suppressor cells; Hepatic tolerance; Immunotherapy

Core tip: The liver is an immune privileged organ that contains cells exhibiting powerful immune regulatory activity. Hepatic stellate cells (HpSCs) possess weak antigen-presenting ability and function as immunological bystander cells in the regulation of the immune response by the way of induction of effector T cell apoptosis and the generation of myeloid-derived suppressor cells and regulatory T cells. The combination of these mechanisms indicates that HpSCs are a potent immunoregulatory entity capable of modulating immune responses in the liver. HpSCs can orchestrate both innate immunity and adaptive immunity to build a negative immune network that

leads to immune tolerance. Further understanding of hepatic immune tolerance will provide the basis for developing new immunotherapies that target transplant rejection, chronic viral infection and cancer.

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INTRODUCTION

Tolerogenic properties of the liver can be initially established by spontaneous acceptance of liver transplants in many species without the requirement of immunosuppression[1-3]. However, hepatocyte transplants are acutely rejected, apparently resulting from immune attacks, because syngeneic hepatocyte transplants survive indefinitely[4]. This finding suggests that liver nonparenchymal cells (NPCs) play an important role in protecting parenchymal cells from rejection. Yu *et al*[5] studied mouse liver NPCs and found that hepatic stellate cells (HpSCs) have potent immune regulatory activity.

HpSCs are located in the subendothelial space adjacent to the basolateral surface of hepatocytes and make up approximately 15% of the total cell population within the liver. They express very low amounts of major histocompatibility complex (MHC) and co-stimulatory molecules[6]; moreover, HpSCs exhibit low antigen uptake and insufficient processing ability[7,8]. HpSCs are the major site of vitamin A storage within the body[9,10] and have been extensively studied regarding their role in fibrogenesis and induction of cirrhosis. Once activated by an insult, HpSCs become myofibroblasts and deposit extracellular matrix, leading to liver fibrosis[11]. HpSCs are difficult to identify based on surface markers, even though intracellular desmin and α-smooth muscle actin (αSMA) have been used for the identification of quiescent and activated HpSCs, respectively. Nevertheless, a recent publication showed that HpSCs can be differentiated from other liver cells using their size, granularity, and high UV-fluorescence (due to their vitamin A content) in combination with the absence of scavenger activity[12]. This overview will discuss the relationship between HpSCs and immune cells and their participation in hepatic tolerance.

HPSCS AND MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSCs) represent an intrinsic part of the myeloid-cell lineage and are a heterogeneous population that is comprised of myeloid-cell progenitors and precursors of myeloid cells. In healthy individuals, immature myeloid cells (IMCs) generated in bone marrow quickly differentiate into mature granulocytes, macrophages or dendritic cells (DCs). In pathological conditions, such as cancer, various infectious diseases, sepsis, trauma, transplantation or some autoimmune disorders, a partial block in the differentiation of IMCs into mature myeloid cells results in an expansion of MDSCs[13]. In mice, MDSCs express both the myeloid lineage differentiation antigen Gr-1 (Ly6G and Ly6C) and the M integrin CD11b. The equivalent of the MDSC in humans is defined as the $CD14^{-}CD11b^{+}$ $CD33^{+}CD15^{+}$ phenotype or cells that express the CD33 marker but lack the expression of markers of mature myeloid and lymphoid cells and the MHC class Ⅱ molecule HLA-DR[14-16]. However, MDSCs share many unifying features, including high expression levels of the immunosuppressive molecules arginase 1 and inducible nitric oxide synthase (iNOS) and, more importantly, their immunosuppressive function[17].

Cotransplantation of HpSCs with allogeneic islets effectively protects the islet allografts from rejection without the requirement of immunosuppression *via* inhibition of the $CD8^{+}$ T-cell response, enhancement of regulatory T-cells[18,19] (Figure 1), and induction of MDSCs[20]. The role of MDSCs is vital to induction of immune tolerance, and this process occurs by skewing the differentiation and effector function of T cells.

Chou *et al*[20] demonstrated that HpSCs promoted the generation of MDSCs both *in vitro* and *in vivo*. Induction of MDSCs is dependent on an intact interferon gamma (IFN-γ) signaling pathway in HpSCs and is mediated by soluble factors, suggesting that the specific tissue stromal cells play a crucial role in regulating immune responses *via* inflammation-induced generation of MDSCs. One of the effective soluble factors secreted by HpSCs is complement component 3 (C3). C3 deficient HpSCs lose their ability to induce MDSCs and, consequently, fail to protect the cotransplanted islet allografts. HpSCs produce complement activation factor B and factor D, which then enhances C3 cleavage into the activation products iC3b and C3d. Addition of exogenous iC3b leads to differentiation of MDSCs with potent immune-inhibitory function[21]. HpSCs are a major source of the immunoregulatory metabolite all-trans retinoic acid (ATRA) in the liver, which may contribute to the generation of tolerogenic DCs in that location. ATRA has been shown to enhance both Arginine 1 and iNOS expression in DCs, resulting in a tolerogenic phenotype[22]. MDSCs induced by HpSCs express B7-H1 and secrete iNOS, which leads to the protection of islet allografts from rejection when MDSCs are cotransplanted with allogeneic islets. This process is associated with attenuation of CD8 T cells in grafts and marked expansion of regulatory T (Treg) cells, which contribute to MDSC-induced T cell hyporesponsiveness[23,24]. These findings provide novel

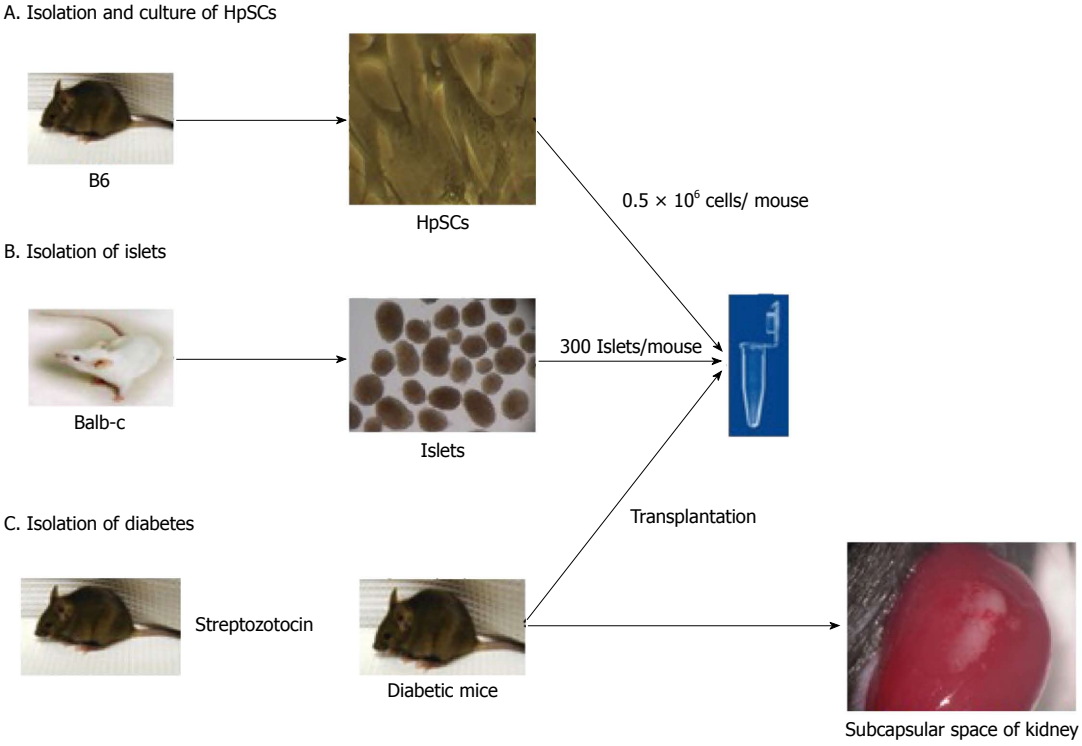

Figure 1 Hepatic stellate cells cotransplanted allogeneic islet animal model. Diabetes was induced in recipients with a single intraperitoneal injection of streptozotocin (220 mg/kg body weight). Only mice with nonfasting blood glucose levels exceeding 350 mg/dL were used as recipients. Islets were isolated from donor pancreas by collagenase V. After separation on a Ficoll gradient, the islets were purified by hand picking. Three hundred freshly isolated islets alone or mixed with 1 or 3 × 10^5 hepatic stellate cells (HpSCs) were aspirated into polyethylene tubing, pelleted by centrifugation for 2 min, and then gently placed under the subcapsular space of the recipient kidney. Transplantation was considered successful if the nonfasting blood glucose returned to and remained normal (< 150 mg/dL) for the first 4 d after transplantation. The tail vein nonfasting blood glucose level was monitored after transplantation, and the first day of 2 consecutive readings of blood glucose levels greater than 350 mg/dL was defined as the date of diabetes onset. No immunosuppressive reagents were administered throughout the experiments.

mechanistic insights into influence of local tissue cells on the differentiation of myeloid cells and may assist in the development of MDSC-based therapy in clinical settings.

IMMUNOTHERAPY

Li *et al*[25] showed that adoptive transfer of HpSC-induced MDSCs successfully reversed disease progression in experimental autoimmune myasthenia gravis (EAMG), a T cell-dependent and B cell-mediated model for myasthenia gravis. In addition to ameliorating the disease severity, MDSC-treated EAMG mice showed suppressed acetylcholine receptor (AChR)-specific T cell responses, decreased levels of serum anti-AChR IgGs, and reduced complement activation at the neuromuscular junctions. MDSCs directly inhibited B cells through multiple mechanisms, including PGE2, inducible NO synthase, and arginase. These results demonstrated that HpSCs induce MDSCs concurrently suppress both T and B cell autoimmunity, leading to effective treatment of established EAMG. Another MDSC-based immunotherapy was performed in hemophilia A mice (factor Ⅷ deficiency)[26]. An adverse effect of factor Ⅷ infusion therapies used for the treatment of hemophilia A is the production of antibodies (inhibitors) against factor Ⅷ, which is a T cell-dependent and B cell-mediated process. HpSC mediated MDSCs, propagated from hemophilia A mice, can also inhibit the proliferation and activation of B cells stimulated by IgM and interleukin-4 (IL-4). Administration of MDSCs, mediated by HpSCs, induced $CD4^+$ T cell and $B220^+$ B cell hyporesponsiveness to factor Ⅷ and reduced inhibitor formation in hemophilia A mice.

A recent study by Dusabineza *et al*[27] revealed that cotransplantation of hepatocytes with HpSCs could improve hepatocyte engraftment *in vivo*. Four weeks after human hepatocytes were cotransplanted with human HpSCs into mouse livers, human albumin positive (huAlb+) hepatocytes formed clusters and were more numerous, occupying 2 to 5.9-fold more surface area on the tissue section than in livers transplanted with hepatocytes alone. Increased huAlb mRNA expression in livers transplanted with the cell mixtures confirmed these results. The presence of HpSCs increased the number of hepatocytes entrapped in the host liver at an early time point post-transplantation but not their proliferation in situ as assessed by the cumulative incorporation of BrdU. Additionally, no accumulation of αSMA+ activated HpSCs or collagen deposition at 4 wk post-transplantation was found. Engrafted hepatocytes also formed E-cadherin+ adherent junctions with adjacent

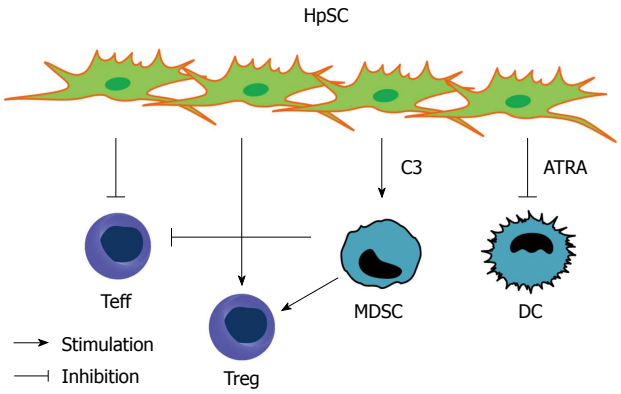

Figure 2 Immune regulatory functions of hepatic stellate cells. Hepatic stellate cells exert potent immune regulatory mechanisms to prevent immense inflammation in the liver. Hepatic stellate cells (HpSCs) can induce regulatory T cells and inhibit effector T cells responses and the function of APCs. These mechanisms contribute to the unique immune tolerant microenvironment of the liver. DCs: Dendritic cells; MDSCs: Myeloid-derived suppressor cells; Treg: Regulatory T cells; Teff: Effector T cells; ATRA: All-trans retinoic acid; C3: Complement component 3.

donor or mouse hepatocytes. Cotransplantation of hepatocytes with HpSCs into a healthy liver recipient does not generate fibrosis but significantly improves the engraftment of hepatocytes, probably by ameliorating cell homing.

Taken together, HpSC-based cell therapy has been successfully conducted based on the above studies, and the results increase the therapeutic potential of this technique.

HPSCS AND T CELLS

HpSCs play an important role in the regulation of sinusoidal blood flow (contractility), maintenance of hepatic architecture (extracellular matrix synthesis), and production of various growth factors. During liver injury, HpSCs transform into myofibroblast-like cells and deposit extracellular matrix, causing liver fibrosis. Activated HpSCs also recruit lymphocytes by producing chemokines[28], and they produce numerous cytokines with either proinflammatory or anti-inflammatory activity[29-31].

HpSCs have been reported to be non-professional antigen-presenting cells (APCs) that do not constitutively express the MHC class Ⅱ proteins required for interaction with naive T cells; these are expressed only upon stimulation by certain cytokines such as IFN-γ.

HpSCs stimulated by IFN-γ express MHC class I and costimulatory molecules and upregulate the expression of IL-6, transforming growth factor-β, and migration inhibitory factor. Activated HpSCs markedly expresses B7-H1 and then induce the T-cell hyporesponsiveness associated with enhanced T cells apoptosis. Blockade of B7-H1/PD-1 ligation significantly reduces HpSC immunomodulatory activity in mice and humans[5,32]. The *in vitro* evidence of immune modulatory activity of HpSCs was validated *in vivo* in an islet transplantation model. Cotransplanted HpSCs that effectively protected islet allografts from rejection formed a multi-layered capsule, which reduced allograft immunocyte infiltrates by enhancement of apoptotic death[18]. Yang *et al*[33] noted that expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an apoptosis-inducing ligand, on HpSCs was crucial in the protection of islet allografts because HpSCs derived from TRAIL knockout mice demonstrated less inhibitory activity towards T cell proliferative responses and substantially lost their capacity to protect cotransplanted islet allografts from rejection. This finding may reflect an intrinsic mechanism of immune inhibition mediated by liver-derived tissue cells. Jiang et al demonstrated that HpSCs selectively expanded regulatory T cells ($CD4^+CD25^+FoxP3^+$) in an interleukin-2-dependent manner[34]. These expanded regulatory T cells successfully inhibited T cell proliferation in responses to an anti-CD3 monoclonal antibody or alloantigens in a nonspecific major histocompatibility complex manner.

The relationship between HpSCs and B cells is not fully understood. Brandão *et al*[35] conducted a study to verify the relationship between plasma cells and HpSCs in human autoimmune hepatitis. They found a significant correlation between HpSCs, the score of fibrosis and the number of plasma cells. Additionally, they found a co-localization of plasma cells and HpSCs. Patients who had successfully been treated were found to have a reduced number of both cells. The result suggests a direct interaction between the HpSCs and plasma cells, but the exact mechanism is still unknown.

CONCLUSION

It is not surprising that the liver, as an immune privileged organ, contains cells that exhibit powerful immune regulatory activity[36]. Due to its anatomical location and function, the liver is continuously exposed to various antigens, including dietary and commensal antigens. In the long journey of evolution, the liver has acquired the ability to control inappropriate immune responses to those harmless antigens. HpSCs possess weak antigen-presenting ability and function as immunological bystander cells in regulating immune responses *via* the induction of effector T cell apoptosis and generation of MDSCs and Treg cells[5,18-20]. The combination of these mechanisms indicates that HpSCs are a potent immunoregulatory entity capable of modulating immune responses in the liver (Figure 2).

Liver sinusoidal endothelial cells (LSECs) are another abundant NPC population in the liver and line the hepatic sinusoids. LSECs represent the unique characteristics of APCs and have an increased scavenger ability that is different from HpSCs. LSECs can also restrain inflammation by induction of Treg cells[37], as evidenced by *in vivo* studies in a mouse model of multiple sclerosis and experimental autoimmune encephalomyelitis[38]. LSECs are capable

of cross-presenting circulating tumor antigens, thereby inducing non-responsive antigen-specific $CD8^+$ T cells through the PD-1/PD-L1 pathway[39]. LSECs can also negatively regulate the APC function of neighboring DCs, which lose their ability to prime naïve T cells by downregulating DC costimulatory signals[8]. However, HpSCs are potentially more beneficial for conducting immunotherapy than LSECs because HpSCs can produce large amounts of MDSCs that are broadly distributed to primary and secondary lymphoid organs to exert their immune-modulatory ability and induce immune tolerance.

Organ transplantation has been widely applied for decades, but cellular transplants, including islets and hepatocytes, remain largely experimental because the side effects of immunosuppression often outweigh the benefits. Therefore, it is crucial to develop a protocol to reduce or avoid immunosuppression. The recent studies related to allogeneic islets cotransplanted with HpSCs or MDSCs mediated by HpSCs show that these cells achieve long-term survival without immunosuppressive medications in mice and offer a promising future for clinical application.

In summary, activated HpSCs can orchestrate both innate immunity and adaptive immunity to build a negative immune network that leads to immune tolerance. Further understanding of hepatic immune tolerance will provide the basis for developing new immunotherapies that target transplant rejection, chronic viral infection and cancer.

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TOPIC HIGHLIGHT

2015 Advances in Alcoholic liver disease

Advances in alcoholic liver disease: An update on alcoholic hepatitis

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Abstract

Alcoholic hepatitis is a pro-inflammatory chronic liver disease that is associated with high short-term morbidity and mortality (25%-35% in one month) in the setting of chronic alcohol use. Histopathology is notable for micro- and macrovesicular steatosis, acute inflammation with neutrophil infiltration, hepatocellular necrosis, perivenular and perisinusoidal fibrosis, and Mallory hyaline bodies found in ballooned hepatocytes. Other findings include the characteristic eosinophilic fibrillar material (Mallory's hyaline bodies) found in ballooned hepatocytes. The presence of focal intense lobular infiltration of neutrophils is what typically distinguishes alcoholic hepatitis from other forms of hepatitis, in which the inflammatory infiltrate is primarily composed of mononuclear cells. Management consists of a multidisciplinary approach including alcohol cessation, fluid and electrolyte correction, treatment of alcohol withdrawal, and pharmacological therapy based on the severity of the disease. Pharmacological treatment for severe alcoholic hepatitis, as defined by Maddrey's discriminant factor $\geqslant$ 32, consists of either prednisolone or pentoxifylline for a period of four weeks. The body of evidence for corticosteroids has been greater than pentoxifylline, although there are higher risks of complications. Recently head-to-head trials between corticosteroids and pentoxifylline have been performed, which again suggests that corticosteroids should strongly be considered over pentoxifylline.

Key words: Alcoholic Hepatitis; Maddrey discriminant function; Corticosteroids; Pentoxifylline; Alcoholic liver

disease

Core tip: Alcoholic hepatitis is a pro-inflammatory chronic liver disease that is associated with high short-term morbidity and mortality in the setting of chronic alcohol use. Management consists of a multidisciplinary approach including alcohol cessation, fluid and electrolyte correction, treatment of alcohol withdrawal, and pharmacological therapy based on the severity of the disease. Pharmacological treatment for severe alcoholic hepatitis, as defined by Maddrey's discriminant function ⩾ 32, consists of either prednisolone or pentoxifylline for a period of four weeks. The body of evidence in favor of corticosteroids has been greater than pentoxifylline, although there are higher risks of complications.

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INTRODUCTION

Alcoholic hepatitis (AH) is a life threatening complication of alcoholic liver disease and presents with a mortality rate of up to 25%[1-6]. AH was first described in 1961 by Dr. Gordon Beckett, who noted several patients with constitutional symptoms followed by worsening jaundice, death in the setting of significant alcohol intake. In general, patients with a diagnosis of AH present in their fifth to sixth decade of life[2-4]. Amount of alcohol intake is the single most important predictor of severity of AH. While reports vary, current consensus is that patients with AH typically ingest over 100 to 120 g of ethanol on a daily basis for 10 to 20 years, with a standard drink equal to 14 g of pure alcohol, which is equivalent to 12 ounces (354.88 mL) of beer, 5 ounces (147.87 mL) of wine, 1.5 ounces (44.36 mL) or a "shot" of 80-proof liquor[2-5]. A study by Bellentani *et al*[5] demonstrated an odds ratio of 13.7 for cirrhosis and 23.6 for non-cirrhotic liver disease in patients with an alcohol intake greater than 30 g per day compared to nondrinkers. Other risk factors for the development of AH include gender and genetic factors. For instance, women have a higher risk of developing alcoholic liver disease relative to men[6]. In addition, an elevated body mass index with or without other components of metabolic syndrome and complicated by non-alcoholic fatty liver disease can lead to synergistic hepatic dysfunction in patients with coexisting alcoholic liver disease[4]. Abstinence from alcohol use is the most fundamental step to successfully treat AH, as demonstrated by a marked reduction in liver-related deaths during the alcohol prohibition era from 1920 to 1933 in the United States. Besides alcohol cessation, the management of AH includes treatment of alcohol withdrawal, correcting electrolyte and fluid abnormalities, optimizing nutritional status and assessing candidacy for pharmacological interventions. Severe AH, as defined by Maddrey's discriminant function ⩾ 32 has been associated with poor prognosis with one-month mortality ranging between 25% to 35%. Despite clinical evaluation of numerous pharmacologic agents, only two, corticosteroids and pentoxifylline have been recommended by practice guidelines[3]. There is no consensus regarding the preferred, first-line treatment option for AH. Nonetheless, early recognition leading to prompt diagnosis, presence or absence of cirrhosis and severity of AH are the key determinants of outcome.

PATHOPHYSIOLOGY OF ALCOHOLIC HEPATITIS

The alcohol-induced hepatic steatosis can largely be attributed to impairment of fatty acid oxidation through the expression of cytokines, reactive oxygen species, oxidative stress, and increased activity of lipogenic enzyme regulators including sterol regulatory element binding proteins (SREBPs) and SREBP-1[7]. The most distinctive histologic feature of AH compared with other forms of hepatitis is predominantly neutrophilic inflammation. This has led to a wide spectrum of proposed theories regarding the pathophysiology of AH. The increased number of neutrophils may be reflective of the increased presence of cytokines, reactive oxygen species, and proteases within the hepatocytes. It has been suggested that pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8 are activated and secreted by Kupffer cells and other inflammatory cells (neutrophils and macrophages) following chronic alcohol exposure[8,9]. In particular, IL-8 is well known for its chemotaxis activity, which may explain the increased presence of neutrophils. Other inflammatory cytokines including TNF-α, IL-1 and IL-6 are activated by the release of free radicals and oxygen reactive species induced by oxidative stress within hepatocytes and Kupffer cells. These inflammatory cytokines likely contribute to the pathophysiology of alcoholic liver disease as suggested by several studies[8,9]. Iimuro *et al*[8] treated ethanol-fed mice with TNF-α antibodies. A reduction in the degree of hepatic inflammation and necrosis was noted in mice treated with TNF-α antibodies. In addition to TNF-α, cyclooxygenase 2 (COX-2) has also been shown to be elevated in mice fed with ethanol[9]. COX-2 is an enzyme involved in the production of prostaglandins and thromboxane. Thus, raising the possibility of COX-2 inhibitors as a potential therapeutic

target[9]. Other potential markers associated with AH include leukotriene B4 and lipopolysaccharides[10,11].

The importance of an alcohol-induced increase in gut permeability as a mechanism underlying endotoxemia leading to activation of inflammatory cytokines is increasingly being recognized[12]. Whether the increase in gut permeability is primarily gastroduodenal or intestinal remains under debate. However, evidence suggests that the increase in gut permeability following acute alcohol ingestion is longer lasting among patients with advanced liver disease, compared to normal controls[12].

Alcohol is normally metabolized to aldehyde by the enzyme alcohol dehydrogenase (ADH), which is located in hepatocytes and gastric mucosa. ADH is a dimeric molecule composed of different subunits, which may explain the variations in blood alcohol levels in individuals who consume similar amount of alcohol. Aldehyde is further oxidized to acetate through the enzymatic activity of aldehyde dehydrogenase (ALDH) within the mitochondria. ALDH is also composed of multiple isoforms, which may explain the flushing phenomenon noted in 75% of Asians and is associated with the accumulation of aldehyde[13]. In chronic alcoholics, the overabundance of ethanol leads to high enzymatic activity of ADH and ALDH, along with induction of the microsomal enzyme oxidation system, in particular the cytochrome CYP2E1[14]. Both enzymes are predominantly located along the centrilobular portion of the hepatocytes, which is often the initial area of inflammation and fibrosis. The high enzymatic activity of cytochrome CYP2E1 causes increased production of reactive oxygen species, leading to chronic inflammatory changes as a result of oxidative stress. Aldehyde itself can also act as a highly reactive molecule, forming covalent bonds with amino acids attached to hepatocellular membrane, forming adducts that can be antigenic[15]. Presence of adducts can trigger a cell-mediated cytotoxic reaction that can lead to hepatocellular damage. In addition to its immunologic effects, formation of adducts can disrupt epithelial tight junctions leading to increased permeability within hepatocytes and gastrointestinal tract.

HISTOPATHOLOGY OF ALCOHOLIC HEPATITIS

The histological features associated with alcohol-induced hepatocellular injury can be categorized into three distinct findings: alcoholic fatty liver (steatosis), alcoholic hepatitis and alcoholic cirrhosis[16-21]. Hepatic steatosis, or fatty liver as a result of alcohol consumption is typically benign and reversible, but can rarely lead to lobular or perivenular fibrosis and death[17-19].

Several factors including cytokines and alcohol byproducts as noted in the previous section contribute to the development of AH-related steatosis, inflammation and fibrosis. It is important to note that hepatic steatosis, inflammation and fibrosis can often coexist in patients with AH. Histologically, AH is characterized by a wide spectrum of changes including micro- and macrovesicular steatosis, acute inflammation with predominantly neutrophilic infiltration, hepatocellular necrosis, perivenular and perisinusoidal fibrosis, and characteristic eosinophilic fibrillar material (Mallory's hyaline bodies) noted in ballooned hepatocytes. Although, traditionally Mallory's bodies have been associated with AH, these findings are not specific and can be seen in other forms of chronic liver disease. As noted earlier, the presence of focal intense lobular infiltration of neutrophils is what typically distinguishes AH from other forms of hepatitis, in which the inflammatory infiltrate is primarily composed of mononuclear cells. Typically, neutrophilic infiltration of perivenular region (zone 3) is noted during the early stages of AH. As the disease progresses, the histological changes expand towards the portal tracts. Alcoholic cirrhosis develops as a result of longstanding alcohol use and is characterized by collagen deposition around the central hepatic vein and along the sinusoids, resulting in a characteristic "chicken-wire" pattern of fibrosis and micronodular cirrhosis. On the other hand, patients with alcoholic cirrhosis who become abstinent develop macronodular cirrhosis due to the absence of alcohol and its anti-proliferative effect.

CLINICAL MANIFESTATIONS OF ALCOHOLIC HEPATITIS

Alcoholic hepatitis is a pro-inflammatory clinical syndrome resulting in a wide range of clinical manifestations. Patients with AH often present with long-standing history of alcohol use (> 100 g per day for 10 to 20 years). Typically, patients with AH present with an acute onset of symptomatic hepatitis with nonspecific symptoms such as anorexia, nausea, vomiting, right upper quadrant pain, proximal muscle wasting, and fever. In particular, excessive alcohol consumption is associated with the highest risk of developing AH. Some patients report drastic increase in their alcohol consumption secondary to recent life stressors such as a divorce, death of a loved one, and loss of employment. Once clinical symptoms develop, many patients develop aversion to alcohol and may discontinue alcohol use even up to several weeks prior to presentation, commonly resulting in misdiagnosis. Therefore, it is important to have an understanding of these salient clinical features of AH and to obtain a thorough history with a focus on timing of signs and symptoms of AH[22-25]. The most common symptoms that lead patients to seek medical care include acute onset of worsening abdominal distension and icterus. Physical examination is notable for jaundice, hepatic encephalopathy, ascites, and

tender hepatomegaly. The tender hepatomegaly is due to the combined effects of hepatocyte swelling in setting of steatosis and stretching of hepatic capsule with subsequent stimulation of nociceptors resulting in painful sensation. Additionally, a bruit can occasionally be appreciated over the liver as a result of increased hepatic blood flow[22]. Other nonspecific symptoms of AH include fever and leukocytosis with neutrophil predominance, which may reflect a potential infection, such as spontaneous bacterial peritonitis especially in the setting of ascites, can occur solely due to AH. While it is worthwhile to rule out infectious sources given degree of immunosuppression from malnutrition, it is also important to understand that fever and leukocytosis may be commonly seen in the presentation of alcoholic hepatitis. Nevertheless, the importance of evaluating for infection among patients with AH cannot be understated as at least a quarter of patients have coexistent infections[26]. Specifically, nonresponse to steroids is most predictive of infection and worse survival in this patient population[26].

Neutrophil predominance has also been noted in liver biopsies of patients with alcoholic hepatitis. Extremely high leukocyte counts (leukemoid reactions) have rarely been seen with alcoholic hepatitis and is associated with high mortalities[25]. Other clinical features include ascites that may be due to transient elevation in portal pressure from hepatocyte swelling or from portal hypertension secondary to cirrhosis. In addition, hepatic encephalopathy and hepatorenal syndrome, both of which are poor prognostic indicators and can still occur in patients with AH in the absence of cirrhosis. Many patients with AH develop noticeable malnutrition. According to one study, malnutrition is noted in greater than 90% of patients with AH[23]. Malnutrition may manifest clinically as temporal muscle wasting, proximal muscle atrophy, and generalized weakness. Malnourishment can be evident through laboratory findings including macrocytosis, which is suggestive of longstanding folic acid and cobalamin deficiency triggered by alcohol consumption.

The most commonly noted laboratory abnormality associated with AH is an elevated bilirubin level. Other abnormalities in laboratory tests include moderately elevated transaminases (usually less than 300 IU/mL) with an AST to ALT ratio > 2; elevated gamma-glutamyl transpeptidase (GGT); leukocytosis (with neutrophil predominance); macrocytosis (due to primary bone marrow hypoplasia from folic acid/cobalamin deficiency and alcohol toxicity); thrombocytopenia (due to primary bone marrow hypoplasia and splenic sequestration); and coagulopathy due to impaired production of coagulation factors and malnutrition resulting in poor vitamin K intake. The ratio of ALT to AST is more specific for alcoholic liver disease due to hepatic deficiency of pyridoxal 5'-phosphate in alcoholic liver disease, which serves as a cofactor for ALT[23]. ALT plays a key role in the intermediate metabolism of glucose and amino acids. The lack of pyridoxal 5'-phosphate or activated vitamin B6 leads to a decreased expression of ALT. In other words, the AST to ALT ratio is primarily due to inappropriate increase in ALT rather than an increase in AST.

DIAGNOSIS OF ALCOHOLIC HEPATITIS

The diagnosis of AH is made by comprehensive evaluation of clinical manifestations and laboratory data in a patient with history of alcohol consumption. A dedicated and thorough history-taking may provide the earliest clinical clues of underlying alcoholic liver disease. Most patients with AH may not be forthcoming due to the social stigma associated with the diagnosis of alcoholic liver disease. Therefore, patients may deemphasize the duration and amount of alcohol intake. Occasionally, discussions with family members and friends may provide a more accurate history and details regarding alcohol consumption. Due to the nonspecific nature of clinical and laboratory findings in alcoholic liver disease, appropriate serological, virological, immunological and genetic tests should be performed to exclude other known etiologies of liver disease. Acetaminophen toxicity, acute viral hepatitis, ischemic hepatitis, Budd-Chiari syndrome, autoimmune hepatitis, or drug-related liver injury can be ruled out by carefully reviewing the past medical history (medications, allergies, social history, travel history, family history, *etc*.), chronology of clinical manifestations, pattern of abnormalities in liver function tests (liver enzymes, total bilirubin and hepatic synthetic function), toxicology screen and diagnostic studies[27]. In patients with coexisting liver disease or unclear history of alcohol consumption, it is may be helpful to confirm the diagnosis of AH with a liver biopsy. Up to 20% patients who are initially suspected of alcoholic liver disease demonstrate evidence of nonalcoholic causes of liver disease on liver biopsy[28]. These clinical scenarios may underscore the importance of a liver biopsy in this patient population to confirm the diagnosis of AH before starting therapy with immunosuppressive agent, such as corticosteroids. Due to the increased risk of bleeding in patients with coagulopathy, a transjugular liver biopsy may provide the safest approach. Finally, a liver biopsy can provide prognostic information regarding the reversibility of alcoholic liver disease by characterizing the extent and severity of underlying histologic damage[28,29].

TREATMENT OF ALCOHOLIC HEPATITIS

Early recognition and diagnosis is crucial in optimizing the management of AH. The treatment of AH requires a multidisciplinary approach that focuses on alcohol cessation, psychosocial evaluation, pharmacologic therapy of AH and treatment of other complications of alcoholic liver disease including withdrawal and nutritional support. Patients should also be considered

for referral to an alcohol and drug rehabilitation center prior to discharge from the hospital.

BEHAVIORAL TREATMENT

Abstinence

Alcohol abstinence is the most essential step towards optimizing the management of patients with AH. Alcohol-induced liver damage without evidence of cirrhosis may actually be reversible with alcohol abstinence[30]. However, the rate at which the liver enzymes return to normal has been variable, typically ranging from a few weeks to several months. Data from nonrandomized trials and retrospective analyses have supported the role of abstinence in patients with alcoholic liver damage[31-34]. It was noted that the five-year survival rate for patients with compensated cirrhosis who abstained from alcohol was 63% to 90% *vs* 41% to 70% in those with continued alcohol use[32,33]. Most liver transplant centers require a 6-month period of documented alcohol cessation prior to initiating an evaluation for liver transplantation.

Interventions including motivational interviewing, cognitive behavioral therapy, and peer support groups have been employed in an effort to maintain compliance. Studies have suggested that there is a small but significant beneficial effect; the actual benefit of these interventions is unclear as data on abstinence maintenance from alcohol use are poor[35]. Therefore, several agents have been studied in order to help maintain abstinence, including naltrexone, nalmefene, acamprosate, and baclofen. Chronic alcohol use results in a change of baseline balance between neuro-excitatory and inhibitory pathways leading to a net neuronal hyperexcitability[36]. Acamprosate (*n*-acetyl-homotaurine), a structurally similar molecule to excitatory amino acids, blocks the neuronal hyperexcitability pathway by acting as a competitive inhibitor[37-39]. The opioid antagonist, naltrexone, exerts an inhibitory effect on the center for alcohol craving in the central nervous system resulting in reduction in alcohol consumption[40]. Nalmefene is another opioid antagonist that has been shown to reduce the amount of alcohol intake[41]. Nalmefene has demonstrated several favorable properties compared to naltrexone including longer duration of action, absence of dose-dependent liver injury, and broader action on central nervous system opiate receptors[41-44]. Baclofen, a gamma-aminobutyric acid agonist acts on the central nervous system and inhibits alcohol craving[45]. Baclofen is the only anti-craving medication to date that has been studied in the context of advanced liver disease[46]. Larger clinical trials are needed to reproduce and confirm the efficacy of these medications.

NUTRITIONAL TREATMENT

Nutrition replacement therapy

As noted above, over 90% of patients with alcoholic hepatitis are also found to have significant malnutrition[47]. Chronic alcohol use induces a profound catabolic state by suppression of appetite, leading to poor oral intake of essential micro- and macronutrients. Additionally, the risk of death from alcoholic hepatitis has been shown to be closely associated with degree of malnutrition[47,48]. Significant protein-calorie malnutrition (PCM) can be commonly seen in these patients, particularly those with jaundice and hepatic encephalopathy[2,49]. Despite the theoretical risk of worsening hepatic encephalopathy, the benefits of an increased protein diet outweigh the risk of hepatic encephalopathy given the degree of malnutrition.

Numerous clinical trials have been performed to further evaluate the impact of nutritional replacement therapy[2,50-54]. One randomized, and controlled trial compared enteral tube feedings to prednisolone therapy for four weeks in patients with severe alcoholic hepatitis. The survival rate between the two groups was similar after 28 d and after one year[55]. However there have been other studies which showed a high variability of results, likely due to the high variability with severity of hepatic decompensation and malnutrition[51-53]. In general, the degree of malnutrition is clearly associated with the response to nutritional replacement therapy. Hence, an aggressive nutritional replacement therapy should be considered for patients with PCM and evidence of severe hepatic decompensation[2]. It is worth noting that although some of these studies have shown improvement in biochemical tests and nutritional status, only a few of them have actually demonstrated any survival benefit[53,54].

Branched-chain amino acids

Many patients require significant protein nutritional support. However, protein ingestion may theoretically increase the risk of hepatic encephalopathy. Despite this risk, the use of specific supplements, in particular the branched-chain amino acids (BCCA), has produced some promising results[56-58]. The rationale for using BCCA as supplementation in patients with alcoholic cirrhosis is to provide protein calories that can be metabolized without the use of the liver. In patients with alcoholic hepatitis, hepatic enzymatic activity may be compromised, leading to an inability to extract maximal calories for the human body. The use of BCCA allows for improved utilization of protein calories. Thus BCCA may improve nutritional status, fulfill metabolic needs, and decrease the risk of hepatic encephalopathy[56]. Despite the rationale for BCCA, there appears to be no advantage in most patients[57,58].

Table 1 Scoring systems for evaluating severity of alcoholic hepatitis

Scoring system	
Maddrey DF	The DF, based on the prothrombin time and total bilirubin, is most commonly used in the decision to treat AH. The DF is a prognostic model at baseline, or static model, similar to MELD and GAHS
MELD	The MELD score, calculated from creatinine, total bilirubin, and international normalized ratio, is classically used for liver transplantation waitlist prioritization but can also be applied as a prognostic indicator in AH
GAHS	The GAHS is calculated based on age, white blood cell count, blood urea nitrogen, total bilirubin, and prothrombin time. The GAHS is another static model that can identify patients at high risk for short-term mortality
Lille score	The Lille model is a dynamic model, which includes the baseline total bilirubin level and the total bilirubin seven days into treatment. Other variables included in the model are age, albumin, creatinine, and prothrombin time. The Lille model is most accurate among these scoring systems in identifying the degree of response to therapy in AH[97]

AH: Alcoholic hepatitis; DF: Discriminant function; MELD: Model for end-stage liver disease; GAHS: Glasgow alcoholic hepatitis score.

Furthermore, clinicians should not restrict protein intake in patients with alcoholic hepatitis even in the setting of hepatic encephalopathy[59]. BCCA supplementation should only be considered in the setting of worsening encephalopathy while on a protein rich diet despite lactulose treatment[59].

PHARMACOLOGICAL TREATMENT

Corticosteroids

There have been numerous studies published that have analyzed the effects of corticosteroid treatment on patients with alcoholic hepatitis. The purpose for corticosteroid therapy is to suppress the inflammatory cytokine cycle[60,61] that predisposes the propagation of liver disease through chronic inflammation. Corticosteroids have been shown to suppress cytokine production, interfere with adduct formation, and inhibit collagen production, thereby reducing hepatocellular injury[62,63]. Corticosteroid therapy, primarily prednisolone, is one of the therapies recommended by the American Association for the Study of Liver Disease and the European Association for the Study of the Liver. Regarding treatment, prednisolone is typically preferred over prednisone as prednisone requires hepatic conversion to the active form prednisolone. This conversion process may be impaired in patients with alcoholic hepatitis. Treatment course typically consists of prednisolone 40 mg per day for 28 d, followed by a taper for 2 to 4 wk.

Numerous clinical trials including randomized and meta-analyses have addressed the role of corticosteroid use in alcoholic hepatitis[49,64-76]. These include studies that demonstrated no improvement in outcomes compared to the control group[49,69], along with those that showed corticosteroid therapy conferred an improvement in short-term mortality in those with severe alcoholic hepatitis. However, the studies that revealed no difference in mortality compared to placebo included patients with differing severities of alcoholic hepatitis. A 2008 Cochrane meta-analysis was performed on 15 randomized trials that compared glucocorticoid therapy with placebo, which revealed a trend towards mortality benefit that did not reach statistical significance. However subgroup analyses involving severe alcoholic hepatitis [Maddrey's discriminant function (DF) $\geqslant$ 32] (Table 1) revealed that there was a reduction in 28-d mortality compared to those treated with placebo (20% *vs* 34%)[77]. Additional meta-analyses have also examined mortality benefit with treatment of severe alcoholic hepatitis, including using other models to assess disease severity including Lille score. This meta-analysis demonstrated that treatment with corticosteroids showed mortality benefit in those with Lille score < 0.56 after one week of treatment. Concerns over corticosteroid use center around the risks of complications, including infection, gastrointestinal bleed, encephalopathy, pancreatitis, glucose intolerance, and psychoses.

In summary, multiple practical guidelines suggest the use of corticosteroids in the setting of severe alcoholic hepatitis, provided that there are no contraindications. In particular patients with severe alcoholic hepatitis as defined by Maddrey's DF $\geqslant$ 32 are candidates for corticosteroid therapy. It is also important to ensure reversible causes of hepatic encephalopathy such as infections, fluid and electrolyte abnormalities, sedatives, or gastrointestinal bleeding should be ruled out and treated appropriately. In situations in which the patient has concomitant chronic liver disease or when the diagnosis is in doubt, it may be reasonable to confirm the diagnosis of alcoholic hepatitis with a liver biopsy before committing a patient to corticosteroid therapy. A few studies have suggested that up to one-fifth of patients with an initial clinical suspicion for alcoholic hepatitis lacked histologic evidence of alcoholic hepatitis on liver biopsy. This stresses the importance of establishing a correct diagnosis as certain studies have suggested a 25% reduction in mortality with treatment. It is also important to recognize that even in the setting of an accurate diagnosis, there is still a high mortality in patients receiving steroids[76]. The balance between maximizing the benefits and minimizing the risks associated with corticosteroid use depends on accurate diagnosis and effective patient selection for treatment. Patients with active infections or other comorbidities may have to be managed conservatively as many of these patients were excluded from the studies. With regards to alcoholic cirrhosis, retrospective data from two clinical trials were unable to establish a clinical benefit with corticosteroid use[69].

Pentoxifylline

Pentoxifylline has been considered an alternative treatment for patients with severe alcoholic hepatitis, largely due to its good safety profile along with the lack of other alternative medications to corticosteroids. However, the evidence supporting use of pentoxifylline is much weaker. Pentoxifylline exhibits its effect by inhibiting the production of TNF-α (increased in alcoholic hepatitis) by altering its gene transcription[78]. Initial studies occurred in the early 1990's in which a randomized, double-blind, placebo-controlled trial of pentoxifylline *vs* placebo was performed. Pentoxifylline 400 mg orally three times a day *vs* placebo was compared in 101 patients with severe alcoholic hepatitis. This study revealed a survival benefit in those treated with pentoxifylline compared to placebo (25% mortality *vs* 46% mortality) during the initial hospitalization[22]. Further subgroup analysis suggested that the root of its survival benefit occurred within the population that developed hepatorenal syndrome (50% deaths in treatment group and 92% deaths in placebo group). This study suggested that pentoxifylline may help to decrease risk of hepatorenal syndrome in patients with severe acute alcoholic hepatitis. A follow-up meta-analysis and systematic review also examined whether such a benefit could be duplicated but these studies failed to show a significant effect on survival benefit[79-81]. Nevertheless, there has been an increasing trend for physicians to treat alcoholic hepatitis with pentoxifylline given its good safety profile and concern with prescribing long-term glucocorticoids in patients with alcohol abuse and dependence.

Corticosteroids vs pentoxifylline

Studies have also examined the combination use of corticosteroids with pentoxifylline. To date, the evidence suggests that there is no additive benefit to pentoxifylline comparing glucocorticoids and pentoxifylline with glucocorticoid therapy alone. Studies fail to demonstrate a difference in survival at six months or occurrence of hepatorenal syndrome[82]. However, up until 2014, there have been no significant head-to-head trials between corticosteroids and pentoxifylline. A recent study from Korea directly compared the efficacy of short-term mortality between prednisolone and pentoxifylline. This was an open-labeled non-inferiority study performed at multiple centers. At one month, the survival rate for corticosteroids was greater than the pentoxifylline (88.1% *vs* 75.8%). Other interesting findings included an improved response to therapy with corticosteroids compared to pentoxifylline as assessed by the Lille model, suggesting a non-equivalent treatment efficacy of pentoxifylline compared to corticosteroid use[82]. The Steroids or Pentoxifylline for Alcoholic Hepatitis (STOPAH) trial is a large multi-center, double-blind, randomized trial conducted in the United Kingdom that compared the different treatment arms of corticosteroid, pentoxifylline, and corticosteroid with pentoxifylline in patients with severe alcoholic hepatitis[83]. The primary end point was mortality at 28 d. The mortality at 28 d was 17% in the placebo-placebo group, 14% in the prednisolone-placebo group, 19% in the pentoxifylline-placebo group, and 13% in the prednisolone-pentoxifylline group. Serious infections were noted in 13% of patients treated with prednisolone *vs* 7% in those treated without prednisolone. This study found prednisolone to be associated with a reduction in 28-d mortality; however, the reduction did not reach significance. Another notable finding was that pentoxifylline did not improve survival in patients with severe alcoholic hepatitis compared to the placebo group. Neither treatment arms reflected improvement in 90-d and one year mortality. This study reaffirms that corticosteroids should still be considered over pentoxifylline for the initial course of treatment for severe alcoholic hepatitis as it has only shown benefit for short-term mortality (Figure 1). This also stresses the difficulties involved with treatment of severe alcoholic hepatitis, and reiterates the high morbidity of this disease.

Other pharmacologic agents

Multiple pharmacologic agents have also been studied to determine if there is any improvement with morbidity and mortality to patients with alcoholic hepatitis. These agents include anabolic steroids, propylthiouracil, colchicine, insulin and glucagon, phosphatidylcholine, infliximab and etanercept. Altogether, no strong, conclusive results are available that have prompted a change in the recommended therapies by the American Association for the Study of Liver Disease and the European Association for the Study of the Liver.

Anti-TNF-α antibodies

Anti-TNF-α antibodies were considered among the most promising potential therapies for alcoholic hepatitis. Levels of the cytokine correlated strongly with severity of disease in AH and low levels were associated with liver regeneration[84]. Based on a feasibility study in 20 patients with biopsy-proven severe AH, anti-TNF-α therapy in addition to prednisone was associated with significant reduction in Maddrey's DF at day 28 compared to prednisone alone[85]. However, two larger randomized controlled trials evaluating anti-TNF-α therapy failed to demonstrate benefit and even suggested harm[86,87]. Therefore, use of anti-TNF-α therapy in AH to date remains investigational.

SURGICAL TREATMENT

Liver transplantation

For patients with end-stage liver disease secondary to alcoholic cirrhosis, including those who fail to respond to medical treatment, liver transplantation is the treatment of choice[88,89]. Patients with alcoholic cirrhosis can be considered for liver transplantation

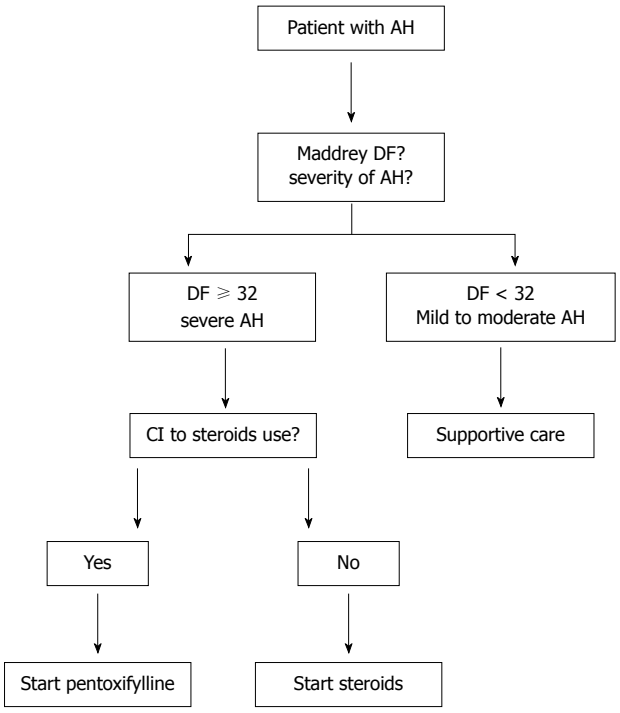

Figure 1 Algorithm to optimize the management of alcoholic hepatitis. DF = 4.6 × [prothrombin time (s) - control prothrombin time] + serum total bilirubin (mg/dL). AH: Alcoholic hepatitis; DF: Discriminant function; CI: Contraindications.

if required listing criteria is met. Multiple factors are evaluated including maintenance of sobriety for at least 6 months, as well as no medical and/or psychosocial contraindications. One issue to consider is whether post-transplant patients are more likely to experience severe recurrent disease. This was noted in a study in which alcohol relapse after liver transplantation was associated with advanced allograft fibrosis and decreased graft survival[90]. Other studies have shown that the duration of pre-transplant abstinence has been a poor predictor for post-transplant sobriety[91-93]. Recurrence of alcohol intake is relatively high following liver transplantation for alcoholic cirrhosis, with some studies suggesting up to 15% patients resume heavy alcohol use, while about 20% to 50% undergo occasional alcohol use[41,50]. Of those patients who underwent liver transplantation, studies show that short-term survival at 1 year was greater than 70%. For highly selective patients, liver transplantation can be life-saving and more cost effective than prolonged medical management of alcoholic cirrhosis[94,95].

FUTURE TREATMENT

In addition to enteral feeds for nutritional support, there are other potential treatments for alcoholic hepatitis. N-acetylcysteine (NAC) has been theorized to exhibit its hepatoprotective effects by acting as an antioxidant. A randomized trial was conducted with 174 patients studying combination of prednisolone with NAC *vs* prednisolone alone[96]. Although the one-month mortality for prednisolone alone was higher compared to prednisolone with NAC (38% *vs* 27%), it did not reach statistical significance. The primary end point for the study looked at six-month mortality, which did not reveal any additional advantage to the addition of NAC to prednisolone *vs* prednisolone alone. Given the finding of improved survival at one-month, additional studies in this direction may be worthwhile.

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TOPIC HIGHLIGHT

2015 Advances in Alcoholic liver disease

Immune dysfunction in acute alcoholic hepatitis

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Abstract

Acute alcoholic hepatitis (AAH) is a serious complication of alcohol misuse and has high short term mortality. It is a clinical syndrome characterised by jaundice and coagulopathy in a patient with a history of recent heavy alcohol use and is associated with profound immune dysfunction with a primed but ineffective immune response against pathogens. Here, we review the current knowledge of the pathogenesis and immune defects of AAH and identify areas requiring further study. Alcohol activates the immune system primarily through the disruption of gut tight junction integrity allowing the escape of pathogen-associated molecular particles (PAMPs) into the portal venous system. PAMPs stimulate cells expressing toll-like receptors (mainly myeloid derived cells) and initiate a network of intercellular signalling by secretion of many soluble mediators including cytokines and chemokines. The latter coordinates the infiltration of neutrophils, monocytes and T cells and results in hepatic stellate cell activation, cellular damage and hepatocyte death by necrosis or apoptosis. On the converse of this immune activation is the growing evidence of impaired microbial defence. Neutrophils have reduced phagocytic capacity and oxidative burst and there is recent evidence that T cell exhaustion plays a role in this.

Key words: Alcoholic hepatitis; Alcoholic liver disease; Toll-like receptors; Gut dysbiosis; T cell exhaustion

Core tip: Acute alcoholic hepatitis (AAH) has high short-term mortality and is challenging to treat with

only glucocorticoids demonstrating proven survival benefit. Development of other effective treatment requires a clear understanding of the mechanisms of immune dysfunction in AAH. Here, we review recent progress in the field and identify areas in need of further research; particularly the role of gut dysbiosis in allowing presentation of pathogen associated molecular patterns to innate receptors on myeloid cells and the subsequent recruitment of immune cell subsets. Recent data demonstrating that T cells have an exhausted phenotype and result in impaired antimicrobial defence is also discussed.

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INTRODUCTION

The United Kingdom has seen an increasing burden of liver related mortality over recent decades with rates increasing 4-fold since the 1970s and 5-fold in the under-65s[1]. This is closely mirrored by the relative affordability of alcohol over this time[2], which is 61% more affordable in 2013 than 1980[3] suggesting that alcohol is an important driving factor for liver disease in the United Kingdom. However, this is not a problem unique to the United Kingdom, similar changes in alcohol consumption and alcohol related mortality have been observed in Northern and Eastern Europe[1] as well as in sub-Saharan Africa, South America and Asia[4].

Given the increasing global consumption of alcohol, it is not surprising that the incidence of acute alcoholic hepatitis (AAH), a serious complication of harmful alcohol use, has also been rising over recent years[5]. AAH is a clinical syndrome characterised by jaundice and coagulopathy in a patient with a recent history of heavy alcohol consumption[6] and has a high short term mortality of up to 40%[7]. It should be clearly differentiated from alcoholic steatohepatitis, a histological diagnosis, which can occur outside the context of current alcohol misuse[8-10].

AAH is increasingly recognised as a systemic inflammatory condition, leading to progressive organ dysfunction and the presence of a systemic inflammatory response syndrome confers a poor prognosis[9]. As well as marked immune activation, there is severe impairment of immune protection against pathogens[11,12]. The discordance between the primed state of the immune system and its failure in microbial defence is yet to be fully explained and an understanding of this dysfunction would certainly help in identifying novel therapeutic targets. To date, therapy has focused on the suppression of an activated immune system and numerous clinical trials have been conducted to evaluate immuno-modulatory (for example, glucocorticoids) or anti-inflammatory therapies [for example, tumour necrosis factor (TNF) alpha antagonists] which target systemic immune activation. Initial promising results from an open label study of infliximab[13] were not confirmed in a randomised controlled trial which was stopped due to excess mortality and infection[14]. Similarly, etanercept treatment was associated with increased mortality[15]. Pentoxifylline, a non-selective phosphodiesterase inhibitor with anti-TNF properties, has also shown no benefit either in combination with glucocorticoids[16,17] or alone[18]. To date only glucocorticoids have a proven short term survival benefit[19,20]. The challenge is how to strike the correct balance between supressing an overactive immune system without further impairing its protective role since death through sepsis remains a significant issue with immunosuppressive treatments[13,21].

The rising incidence of AAH together with its high mortality and limited treatment options has resulted in the European Association for the Study of the Liver identifying AAH as a priority area for research with a specific aim to investigate molecular signals which may predict clinical outcome[22]. Here, we review the current knowledge of the immune mechanisms involved in the pathogenesis of AAH and focus on areas in need of future study. We have not discussed the direct toxic effects on the liver of alcohol and its metabolites including oxidative stress and acetaldehyde adducts which have been reviewed in detail elsewhere[23,24].

THE GUT-LIVER AXIS

The mechanisms by which alcohol activates the immune system were first conclusively elucidated by Thurman *et al*[25] in 1999. The presence of alcohol allowed the presentation of pathogen associated molecular patterns (PAMPs) to hepatic macrophages (Kupffer cells) by modulating intestinal permeability[25]. Subsequent studies have highlighted the importance of the effects of alcohol on the gut microbiome itself with alterations in both the number and balance of organisms which contribute to the breakdown of the intestinal barrier[26]. In a murine model of alcohol related liver disease (ALD), intestinal bacterial overgrowth occurs with a corresponding reduction in probiotic species such as *Lactobacillus*[26]. In humans, bacterial overgrowth has been found in jejunal aspirates from chronic alcohol misusers[27] but the species of bacteria is also important. In a randomised controlled trial (RCT) of *Lactobacillus* and *Bifidobacterium* probiotic therapy in patients with alcoholic psychosis, baseline levels of intestinal probiotic species were lower than healthy controls and short term probiotic treatment significantly improved biochemical indices[28]. Improvement in clinical disease score has also been demonstrated in a small RCT by *Escherichia coli Nissle* treatment of patients

with stable cirrhosis[29]. There is also preliminary data that treatment with probiotics can improve neutrophil phagocytic function in stable cirrhosis with normalisation of phagocytosis after 7 d of treatment with *Lactobacillus casei Shirota*[30].

To date, the most detailed human study of the effects of alcohol on gut dysbiosis compared patients with ALD cirrhosis, patients with alcohol dependence and healthy controls using next generation sequencing techniques to analyse the 16S ribosomal RNA (rRNA)[31]. Compared to the control group a subset of patients displayed gut dysbiosis with significantly lower levels of *Bacteroides* and higher levels of *Proteobacteria*, which was associated with increased systemic endotoxin. Next generation sequencing rRNA studies have yet to be performed in patients with AAH.

Gut dysbiosis alters the intestinal lumen integrity through mechanisms that are incompletely understood. It is clear that the microbiota are key in increasing gut permeability through murine experiments in which the sterilised gut protects against alcohol-induced intestinal barrier leakage[32]. Disruption of tight junctions is probably mediated by microbial metabolism of alcohol to acetaldehyde[33]. However, systemic TNFα and IL-1β, which are increased in patients with AAH, also reduce tight junction integrity so there may be a positive feedback loop in these patients[34]. Furthermore, the loss of probiotic bacterial species may reduce barrier protection since transfer of *Lactobacillus* ameliorates ALD in a mouse model[35]. In a more acute murine model of alcoholic steatohepatitis *Lactobacillus* treatment restored intestinal integrity, reduced oxidative stress and improved histological liver damage[36].

The leaky gut seen in patients with AAH results in presentation of PAMPs to hepatic innate immune cells, particularly Kupffer cells. Chronic alcohol misusers have higher levels of endotoxin [lipopolysaccharide (LPS)] systemically[37] as well as in the portal vein[38] suggesting that there is greater exposure of the liver to microbial components. Interestingly, this defect may be rapidly reversible: in a study of alcohol dependent patients, both intestinal permeability and LPS levels were elevated compared to normal controls but returned to normality after 3 wk of abstinence[39].

Most of our understanding of gut dysbiosis in AAH comes from animal models and patients with chronic ALD. Future study should be directed at analysing the gut microbiome of patients with AAH compared to healthy controls and patients with cirrhosis. A well powered RCT should then be conducted to test the efficacy of specific probiotic therapy designed to restore the microbiome. Work in this area is underway as demonstrated by a single trial registered at Clinicaltrials.gov which is investigating 7 d of treatment with several probiotic regimes but this is not powered to detect survival differences[40]. Prevention of the disruption of gut tight junctions is also an appealing therapeutic target but this is likely to be due to a complex interplay between gut bacteria and innate immunity and a more detailed understanding of the mechanisms of disruption is first required.

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are innate pattern recognition receptors for a wide variety of PAMPs such as microbial components, endogenous molecules and danger signals[41]. The TLR family consists of 10 receptors principally expressed by granulocytes and cells of myeloid lineage. The net action of ligand binding is the activation of the nuclear factor kappa B (NF-κB), activating protein 1 (AP1) and interferon regulatory factor (IRF) families and rapid and robust transcription of pro-inflammatory mediators[42]. In animal models of ALD, increased expression of TLR1, 2, 4, 6, 7, 8, and 9 is reported with increased sensitivity to their respective ligands[43] while in humans, TLR2, 4 and 9 are upregulated in neutrophils from AAH patients[44] suggesting that the TLR signalling pathway is important in the pathogenesis of the disease.

LPS acting *via* TLR4 appears to be the most important interaction in the pathogenesis of AAH. Mice with non-functional mutant TLR4 are protected from alcoholic liver injury[45] as are those with inactivated Kupffer cells[46]. However, both Kupffer cells and non-bone marrow derived liver cells are involved in TLR4-mediated alcoholic liver injury shown by development of ALD in TLR4$^{-/-}$ mice transferred with wildtype bone marrow cells[47]. Furthermore, deficiency in IL-1 receptor associated kinase (IRAK)-M (the negative regulator of TLR4) conferred more severe ALD[48].

The action of LPS and other PAMPs *via* innate receptors on intrahepatic cells initiates a sequence of pro-inflammatory responses. Patients with AAH have elevated levels of pro-inflammatory cytokines mostly produced by myeloid cells including IL-1, IL-6, IL-8 and TNFα (the latter is also related to disease severity)[49-51]. This results in hepatocellular damage *via* TNF Receptor 1 and intrinsic death pathways[52]. In addition, alcohol sensitises Kupffer cells to the effects of LPS[53] and hepatic macrophages and Kupffer cells produce reactive oxygen species in response to chronic alcohol exposure or LPS[54], driving further liver damage.

The TLR system has the potential to be modulated to reduce pro-inflammatory signalling in AAH but still requires more thorough evaluation. TLR expression has been studied in detail in neutrophils from patients with AAH but blockade of the overexpressed TLRs did not result in restoration of normal neutrophil function[44]. The function of other immune subsets with high TLR expression (especially monocytes, macrophages and Kupffer cells) should also be examined.

CHEMOKINES

Stimulation of both immune and non-immune intrahepatic cells results in the secretion of an array of soluble mediators including cytokines and chemokines which co-ordinate the subsequent immune response and determine the balance between liver damage and resolution of inflammation. Chemokines and their respective receptors control the influx of leucocyte subsets into the liver and have been shown to play important roles in shaping the immune response in a variety of liver diseases[55]. Chemokines are low molecular weight proteins which bind to transmembrane receptors triggering a signalling cascade which alters integrin expression allowing interaction with endothelial adhesion molecules. The gradient of chemokine expression increases near the site of inflammation, which ensures the leucocyte is attracted to the appropriate site before migrating through the vascular endothelium.

Interest in chemokines and their receptors as therapeutic targets has increased over recent years since they can control the ingress of specific pro-inflammatory leucocyte subsets into sites of inflammation or injury. Currently, a number of clinical trials evaluating several chemokine and chemokine receptor antagonists for the treatment of inflammatory diseases including asthma, inflammatory bowel diseases and primary biliary cirrhosis have been registered at clinicaltrials.gov. In the context of AAH, many different chemokines have been implicated and the challenge is to determine which pathway to block. Neutrophils may be the most appropriate target since liver tissue from patients with AAH demonstrates a significant neutrophilic infiltration, the degree of which correlates with disease severity[56].

Ischaemic models of acute liver injury demonstrate that neutrophils are activated by TNFα, IL-1β and IL-17 and recruited by CXC chemokines such as CXCL1 (GROα) and CXCL8 (IL-8)[57]. Elevated levels of these chemokines among others have been confirmed in transcriptome microarray and PCR analysis of homogenised liver biopsy material from patients with AAH[58]. Levels of CXCL1, 5, 6 and 8 were all elevated in AAH *vs* normal liver and correlated with neutrophil infiltration and degree of portal hypertension and were associated with a poor prognosis at 90 d[58]. However, the exact role of the neutrophil in the pathogenesis of AAH is unclear[11] and the control of their entry into the liver is not fully understood. Moreover, chemokines that are known to attract neutrophils will also attract other immune cell types. CXCL1, 5, 6, and 8 specifically attract both neutrophils and monocytes which both express the relevant CXCR1 and CXCR2 receptors for these chemokines. Therefore, a clearer understanding of the complex pathways of leucocyte trafficking in AAH is required and blockade of a single component of the pathway may not translate into a clinical benefit[59].

The same transcriptome study also identified CCL20 as being the third most upregulated gene expressed in AAH liver tissue compared to controls[58]. CCL20 binds to CCR6 which is expressed on the Th17 subset of T cells as well as on hepatic stellate cells (HSCs) and $\gamma\delta$T cells and is likely to play an important role in the adaptive immune response.

TH17 CELLS

The Th17 cell subset, defined by its production of IL-17 and expression of RORγt, is derived from naïve $CD4^+$ T cells under the influence of cytokines IL-1β and IL-6[60]. As well as being important in the clearance of extracellular pathogens, it is also implicated in the pathogenesis of several autoimmune[61] and inflammatory diseases including ALD and AAH[62,63]. IL-17 enhances the inflammatory response by stimulating a wide variety of cells including monocytes, endothelial cells and fibroblasts, to secrete CXCL8, a neutrophil chemoattractant[64]. In a positive feedback loop, IL-17 also stimulates CCL20 secretion, itself a Th17 chemoattractant, with high levels of its receptor CCR6 expressed by Th17 cells[65,66].

Th17 cells have been implicated in the pathogenesis of AAH[63]. IL-17 protein in serum from patients with AAH was elevated as was peripheral $CD4^+$ T cell capacity to produce IL-17 on stimulation compared to healthy controls. In AAH liver tissue, there was an enrichment of IL-17^+ cells which were T cells and neutrophils and numbers correlated with degree of fibrosis. Furthermore, it was shown that HSCs have the IL-17 receptor and their secretion of important fibrotic mediators was dependent on IL-17[34].

The liver transcriptome study[58] suggests that in AAH, the high expression of CCL20 results in the infiltration of Th17 cells. Further work has shown that CCL20 levels correlate with clinical severity score, degree of portal hypertension and survival in patients with AAH and, using an animal model of acute on chronic ALD [mice treated with carbon tetrachloride (CCl_4), ethanol and LPS], that macrophages and HSCs are the primary source of CCL20[67]. In addition, exposure of primary HSCs *in vitro* to CCL20 promotes fibrogenesis[67].

Therefore, it is likely that CCL20 mediates hepatic inflammation and fibrosis in AAH by direct effects on HSCs and *via* recruitment of Th17 cells. The Th17 cytokine IL-22 has also been shown to be upregulated in peripheral blood of patients with AH and increased levels subsequently predicted better patient outcome[68]. Plasma IL-17 (but not IL-21 or IL-23) was also elevated compared to healthy controls but not related to outcome. Data was not presented to determine whether IL-17 and IL-22 were co-expressed so it is possible that IL-22 may be from the novel Th22 cells rather than pathogenic Th17 cells and hence may have a hepatoprotective effect. The protective

effects of IL-22 have been demonstrated in a chronic/binge ethanol feeding model where administration of exogenous IL-22 ameliorated liver injury and oxidative stress *via* a STAT3 mechanism[69]. A phase 2 clinical trial of recombinant human IL-22 is currently underway in patients with AAH.

Interestingly, a recent study extensively characterised pathogenic Th17 cells from healthy controls and then confirmed that they were enriched in the peripheral blood and inflamed gut of patients with Crohn's disease[70]. These pathogenic Th17 cells were resistant to steroid-mediated T cell suppression in terms of pro-inflammatory cytokine production and proliferation[70]. An animal model of airways inflammation has also suggested that Th17 cells are steroid resistant[71]. This has been further assessed by gene profile analysis, cytokine expression and proliferation in Th17 cells derived from patients with autoimmune uveitis as well as murine Th17 cells in an experimental model of uveitis, which were shown to be resistant to steroid treatment[72]. The latter study also demonstrated that both human Th17 cells *in vitro* and murine Th17 cells *in vivo* were selectively inhibited by the calcineurin inhibitor, ciclosporin A (CsA)[72].

The observation that Th17 cells are enriched in AAH but may be resistant to steroid treatment may help to explain why some patients do not respond clinically to steroid treatment. Further research is needed to clarify whether these cells are indeed steroid resistant in the context of AAH and whether there are different characteristics in this T cell subset between steroid responders and non-responders. As suggested by the recent *in vitro* and *in vivo* data discussed above[72], rescue therapy with CsA in patients with steroid resistant AAH may be efficacious. It is already well-established therapy for the treatment of steroid resistant acute severe ulcerative colitis albeit with significant toxicities and side-effects[73] but the risk of sepsis with such a potent immunosuppressive agent would be too high to justify a clinical trial in AAH patients unless a method to specifically target Th17 cells could be found. Alternatively, if steroid non-responders could be accurately identified, for example by the bioassay recently reported by our group[74], CsA could be selectively offered to these patients with the highest risk of death from AAH. Another perhaps less toxic approach is to prevent Th17 cell ingress to the liver with anti-CCL20 antibody, which may be particularly efficacious in patients who do not respond to steroid treatment.

T CELL EXHAUSTION

The phenomenon of a primed immune system but with failure of pathogen defence may be caused by a defect in effector cell negative regulatory signalling. Inhibitory pathways exist to maintain immune homeostasis to prevent over-activation and exhaustion of immune cells but allow appropriate clearance of pathogens and tumours. Several pathways exist; the best studied involves the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) family. CTLA-4, a T cell surface receptor, by binding to the same molecules on antigen presenting cells, competitively antagonises CD28, the T cell co-stimulation receptor and prevents T cell activation[75]. Deletion of CTLA-4 in murine models leads to the development of fatal multiorgan autoimmune disease[76], probably as a result of unchecked CD28-mediated T cell stimulation, demonstrating its importance in immune control. A member of the CTLA-4 family, programmed death 1 (PD-1) serves a similar purpose to maintain balance of effector T cell function[77] while T-cell immunoglobulin and mucin domain 3 (TIM-3) also has potent inhibitory functions on both T cells and innate immune cells[78]. It has recently been proposed that inappropriate expression of PD-1 and TIM-3 plays a role in the immune paresis seen in AAH[79].

Impairment in both innate and adaptive immunity was seen in patients with AAH; poor neutrophil antimicrobial function and reduced T cell interferon-γ (IFNγ) production was demonstrated. In addition, PD-1, TIM-3 and their ligands were overexpressed on T cells from the peripheral blood of patients with AAH compared to patients with ALD or healthy controls but when these receptors were blocked the immune defect was overcome. It was shown that the overexpression of these inhibitory molecules was mediated by LPS binding to TLR4 on $CD14^+$ monocytes[79].

These intriguing data inform new paradigms of how an active immune system exposed to many PAMPs can remain impaired at pathogen clearance and suggest that gut dysbiosis is central to the pathogenesis of the disease. However, the effect of other TLR ligands, cytokines and the direct effect of alcohol on negative regulatory molecule expression (PD-1, TIM-3 and others) have not been investigated. It is important to note that this study was conducted using peripheral blood derived immune cells which may not accurately reflect what occurs within the liver and therefore a similar effect needs to be demonstrated on intrahepatic immune cell subsets. Finally, careful consideration of how to translate these findings to a possible therapy is required. Restoration of immune homeostasis involves the rebalancing of pro- and anti-inflammatory pathways. Improving host defence by blockade of these regulatory pathways may result in the tipping of the balance too far in favour of immune activation which may drive further liver damage. An experimental model of AAH should be first employed to assess whether this strategy would have a beneficial effect.

GRANULOCYTE COLONY-STIMULATING FACTOR

The cytokine granulocyte colony-stimulating factor

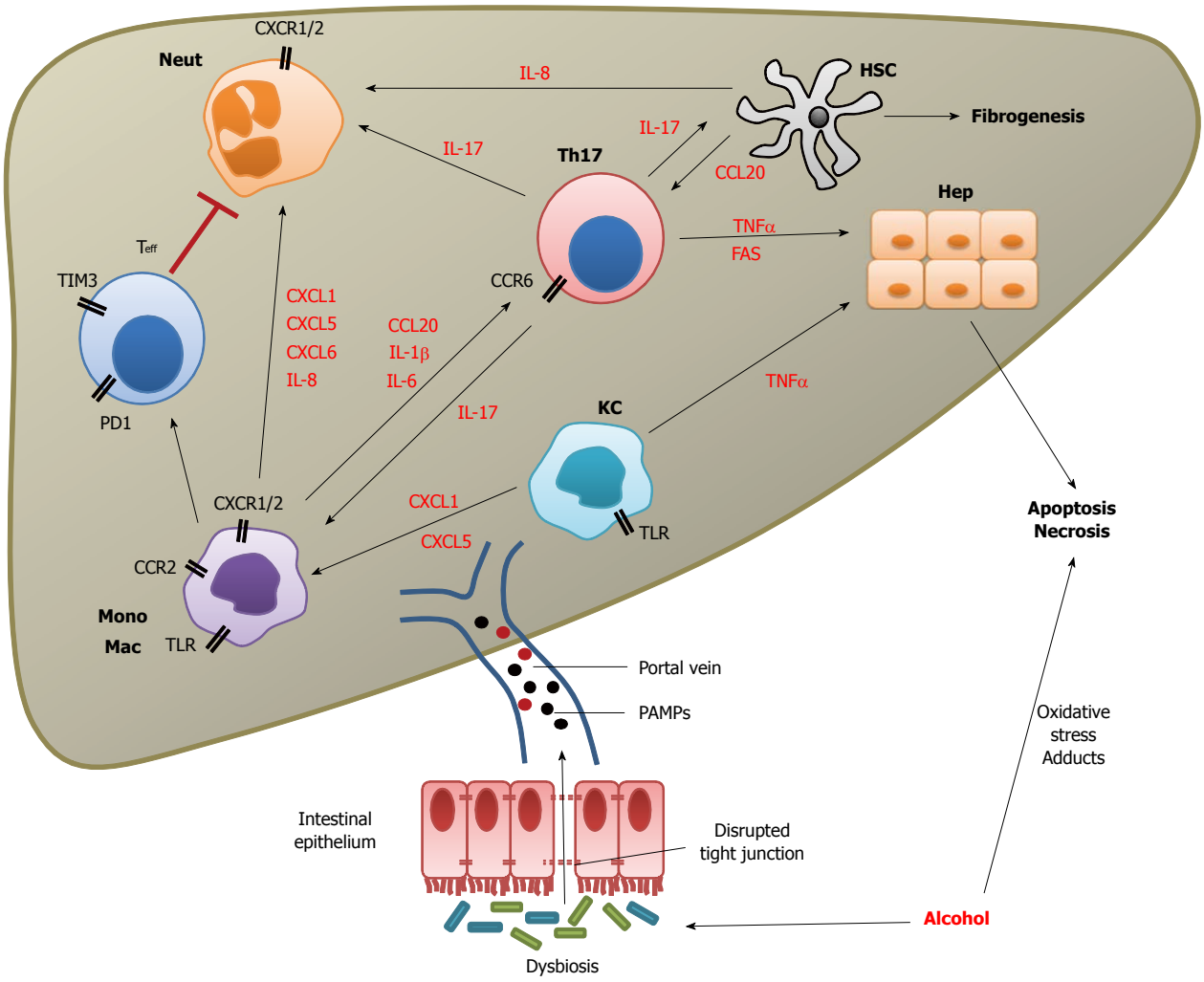

Figure 1 Immune dysfunction in acute alcoholic hepatitis. Alcohol has a direct effect on hepatocytes by production of reactive oxygen species causing oxidative stress. It also results in the production of acetaldehyde adducts which can cause DNA damage, mutagenesis and direct cell death. Alcohol consumption also leads to intestinal bacterial overgrowth and gut dysbiosis with a loss of *lactobacillus* and increase in *proteobacteria* species. Dysbiosis together with the direct effect of acetaldehyde (a metabolite of alcohol) and pro-inflammatory cytokines disrupts epithelial tight junctions and allows the escape of pathogen-associated molecular patterns (PAMPs) into the portal circulation. Within the liver PAMPs are presented to Toll-like receptors (TLRs) on myeloid cells including monocytes (Mono), macrophages (Mac) and Kupffer cells (KC) stimulating release of cytokines and chemokines. In addition, TLR4 activation on monocytes leads to upregulation of negative inhibitory molecules programmed death 1 (PD-1) and T-cell immunoglobulin and mucin domain 3 (TIM-3) on effector T cells (T_{eff}), which in turn inhibit neutrophil (Neut) anti-microbial functions. Chemokines and cytokines coordinate the infiltration and stimulation of other immune cells in particular neutrophils, monocytes (both by CXCL1, 5, 6 and IL-8) and Th17 cells (by CCL20). Th17 cells further increase neutrophil infiltration and also stimulate hepatic stellate cells (HSCs) to produce fibrogenic mediators. TNFα and Fas produced by T cells also leads to hepatocyte cell death by apoptosis through the Fas and TNF receptor pathways.

(G-CSF) stimulates bone marrow production of granulocytes and haematopoietic stem cells and is involved in the proliferation and differentiation of neutrophils but may also play a role in hepatic regeneration[80]. G-CSF treatment enhances the bactericidal and phagocytic capacity of human neutrophils from healthy subjects as well as impaired neutrophils from HIV-1 infected individuals[81]. It is therefore an appealing therapy for AAH which has the potential to both enhance neutrophil function and hepatocyte regeneration.

G-CSF was well tolerated in patients with cirrhosis and alcoholic steatohepatitis; 5 d of treatment was associated with an increase in circulating $CD34^+$ cells (a surrogate for haematopoietic stem cells), increased serum hepatocyte growth factor and proliferation of hepatic progenitor cells in day 7 liver biopsy specimens. However, there was no change in liver function compared to the control group[82]. A randomised open label trial of G-CSF treatment of Acute on Chronic Liver Failure (of which 57% had alcoholic hepatitis as the underlying aetiology) demonstrated increased hepatic $CD34^+$ cells after 28 d and significantly improved 60 d survival[83]. A recent open label RCT of 5 d of G-CSF *vs* standard care (including pentoxifylline) in the treatment of patients with AAH resulted in a greater number of serum $CD34^+$ cells and improved 3 mo survival compared to standard care[84].

These trials have demonstrated the potential benefit of G-CSF in the treatment of AAH but con-

firmation of its benefit in a large double-blind RCT is needed. Further study is required to elucidate the mechanisms of G-CSF action and evaluate its benefit in the context of steroid resistant disease.

CONCLUSION

Evidence drawn from studies on patients with AAH and chronic ALD as well as animal models has enhanced our understanding of the immune mechanisms that occur in AAH. In summary, chronic alcohol consumption leads to gut dysbiosis, disrupting the gut epithelial integrity and allowing the presentation of PAMPs to intrahepatic cells *via* the portal circulation. This in turn causes activation of a network of cells resulting in the alteration of surface molecular patterns and the release of a plethora of soluble mediators, which co-ordinates the influx of immune cells into the liver. In the context of AAH, these immune cell subsets cause direct damage to hepatocytes and stimulate HSCs to produce fibrogenic molecules leading to liver cell death and fibrosis. Additionally, there is evidence of immune paresis with poor innate cell responses and increased T cell exhaustion resulting in a reduced ability to prevent bacterial infection (Figure 1).

Many of the mechanisms of pathogenesis require confirmation and testing in patients with AAH but have the potential to yield new therapeutic targets in the future. Here, we have highlighted the gut microbiome, the expression of TLRs on different myeloid cell subsets, the chemokine pathway, the steroid responsiveness of Th17 cells, T cell exhaustion and G-CSF therapy as priority areas for further research.

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TOPIC HIGHLIGHT

2015 Advances in Alcoholic liver disease

Noninvasive assessment of alcoholic liver disease using unidimensional transient elastography (Fibroscan®)

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Abstract

Unidimensional transient elastography (TE) is a noninvasive technique, which has been increasingly used in the assessment of diffuse liver diseases. This paper focuses on reviewing the existing data on the use of TE in the diagnosis of fibrosis and in monitoring disease progression in alcoholic liver disease, on the factors that may influence the result of fibrosis prediction, and last but not least, on its potential use in assessing the steatosis degree. Therefore, this field is far from being exhausted and deserves more attention. Further studies are required, on large groups of biopsied patients, in order to find answers to all the remaining questions in this field.

Key words: Transient elastography; Alcoholic liver disease; Fibrosis; Steatosis; Liver stiffness; Controlled attenuation parameter

Core tip: This review article summarizes the existing data on the use of transient elastography in the noninvasive assessment of fibrosis and steatosis in alcoholic liver disease and highlights the still open questions in this field.

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INTRODUCTION

Excessive alcohol consumption is a major public health issue[1,2] as it may lead to liver fibrosis and cirrhosis with life threatening complications[3] such as hepatocellular carcinoma (HCC)[4], liver failure and death[5].

The presence and progression of hepatic fibrosis towards cirrhosis is a main prognostic variable, impacting the survival of people with alcoholic liver disease[6]. Consequently, an important goal in alcoholic patients is to reliably identify those with advanced fibrosis and/or cirrhosis, which not only impact the patients' prognosis but may also be used as an argument to support the necessity to quit drinking.

Many efforts have been devoted lately to the development of noninvasive markers and tests that may reliably predict fibrosis stages in chronic liver diseases. One of the newer developments involve ultrasound elastographic methods for noninvasive liver fibrosis assessment, some of which have been studied and developed for the noninvasive assessment of steatosis, as well. The main method discussed here is the unidimensional transient elastography (TE) - Fibroscan®, one of the best studied elastographic methods.

PRINCIPLE

Unidimensional TE is performed using the Fibroscan® equipment (Echosens, Paris) which consists of a 5 MHz ultrasound transducer probe mounted on the axis of a vibrator. Mild amplitude and low frequency vibrations (50 Hz) are transmitted to the liver tissue, inducing an elastic shear wave that propagates through the underlying liver tissue. The velocity of the wave is directly related to tissue stiffness[7].

The technique measures the stiffness in a cylindrical volume 1 cm in diameter and 4 cm in length, amounting to about 1/500 of the entire liver volume - 100 times larger than the volume of the liver biopsy specimen[8,9].

WHO CAN PERFORM THE EXAMINATION?

The measurement can be performed even by a technician after a certain training period (around 100 cases)[9,10]; the clinical interpretation of the results, however, always requires an expert, who can take into consideration the demographics, disease etiology and biochemical profile of the patient at the moment of the examination[8].

PERFORMANCE PARAMETERS OF THE EXAMINATION

In accordance to the producer recommendations, the success rate (the number of measurements required to obtain 10 valid ones) was for a long time limited to at least 60%, while the IQR (interquartile range) to less than 30% of the median (M) liver stiffness (LS)[7], although the best concordance with the biopsy seems to be obtained when its value does not exceed 20% of the median[11].

According to the latest reports, it is considered that the "success rate $\geqslant$ 60%" parameter is no longer necessary, and the examination accuracy depends on the IQR/M ratio, influenced by the median LS value. Three reliability categories are therefore defined, with significantly different diagnostic accuracy: "very reliable" (IQR/M $\leqslant$ 0.10), "reliable" (0.10 < IQR/M $\leqslant$ 0.30 or IQR/M > 0.30 with median LS < 7.1 kPa), and "poorly reliable" (IQR/M > 0.30 with median LS $\geqslant$ 7.1 kPa)[12].

REPRODUCIBILITY

FibroScan appears to have good reproducibility[13]. In a series of 195 patients with chronic liver disease of various etiologies and without ascites, using the FibroScan to identify a suitable portion of the liver for examination, Fraquelli *et al*[14] found that overall agreement between two operators was 0.98 (95%CI: 0.977-0.987), and intraobserver agreement was 0.98 for both operators. Increased body mass index (BMI) (> 25 kg/m^2), steatosis (> 24% of fatty liver cells), and histological evidence of none to mild fibrosis (METAVIR stage < F2) were all significantly associated with reduced interobserver agreement.

LIMITATIONS OF THE TECHNIQUE

Because elastic waves do not travel through liquids, FibroScan has no value in patients with ascites[13]. Another important limitation is the impossibility to examine obese patients[13], because the probe is calibrated for a specific distance between the liver and the chest wall[15] and the low frequency vibration induced by the probe and/or the ultrasound wave can be strongly attenuated by the fatty tissue[7]. Castéra *et al*[15] found that a BMI > 30 kg/m^2 had the strongest association with both test failure and unreliable results. A special probe (XL probe) with a measurement depth of 35-75 mm[16] was developed for morbidly obese patients[17]. A study conducted specifically in patients with a BMI $\geqslant$ 30 kg/m^2 found that the use of the XL specialised probe reduced the rates of failure and unreliable results (LS measurement was successful in 45% of the cases with the M probe, *vs* 76% of the cases with the XL probe)[18].

WHAT ARE THE LS CUT-OFF VALUES FOR THE PREDICTION OF EACH FIBROSIS STAGE IN ALCOHOLIC LIVER DISEASE?

TE assessment of liver fibrosis has already been validated in many people with chronic liver diseases of various etiologies[19-24]. The already published meta-analyses demonstrated that the cause of liver disease is one of the most important factors leading to the heterogeneity of TE results, thus indicating that the different chronic liver diseases should be analysed separately[25-27]. In fact, the cut-off levels for specific stages of hepatic fibrosis vary according to the etiology of the liver disease. This could be easily explained by the fact that LS mainly reflects the amount of liver fibrosis not taking into account its topography and its consequences on liver architecture which are the basis of all semi-quantitative fibrosis staging systems[28].

Compared with other etiologies, few studies have been performed on groups of patients with alcoholic liver diseases (ALD) or, in the studies involving groups of patients with diffuse liver diseases, the ALD cases reach only a small percentage of the entire group[28-38]. The LS cut-off values for fibrosis stage prediction differ quite drastically, mainly due to the presence of inflammation, assessed by transaminase levels[33]. A recent meta-analysis[6], taking into consideration 5 retrospective and 9 prospective studies, with a total of 834 participants, could not identify the optimal cut-off values for the prediction of each fibrosis stage in ALD.

Only one study has established the cut-off values for the prediction of fibrosis stages $\geqslant$ F1 in ALD, namely 5.9 kPa[29], which offered 83% sensitivity and 86% specificity, PPV 97.6%, NPV 35.3% and AUROC 0.84.

For the prediction of stage F2 or above, the TE sensitivity in the studies included in the Pavlov meta-analysis varied from 75% to 100% and the specificity from 80% to 100%, while the cut-off values in the majority of the analysed studies was around 7.5 kPa (range 7.00 to 7.8 kPa)[6]. The following results were obtained when using the 7.5 kPa cut-off in the meta-analysis: sensitivity 0.94 (95%CI: 0.86-0.97); specificity 0.89 (95%CI: 0.76-0.95); positive likelihood ratio (LR+) 8.2 (95%CI: 3.6-18.5); negative likelihood ratio (LR-) 0.07 (95%CI: 0.03-0.17)[6].

In the prediction of stages F3 or above, the sensitivity of the analysed studies in the meta-analysis varied from 72% to 100% and the specificity from 59% to 89% at cut-off values ranging from 8.0 to 17.0 kPa[6]. When considering only the studies yielding LS cut-off values around 9.5 kPa, for the prediction of $\geqslant$ F3 stages, the TE sensitivity varied from 80% to 100% and the specificity from 50% to 80%. In the meta-analysis, when the 9.5 kPa cut-off value was used for the prediction of stages $\geqslant$ F3, the following results were obtained: sensitivity 0.92 (95%CI: 0.83-0.97); specificity 0.68 (95%CI: 0.52-0.80); positive likelihood ratio (LR+) 2.9 (95%CI 1.8-4.5); negative likelihood ratio (LR-) 0.11 (95%CI: 0.05-0.27)[6].

In alcoholic cirrhosis, the median LS value is higher than that observed in patients with viral cirrhosis[29]. This may be explained by the different spatial distribution of alcoholic fibrosis, which develops in centrolobular and perisinusoidal as well as in periportal regions[39]. Hepatic alcoholic lesions are also characterized by liver cell necrosis, reactive inflammation, steatosis and pericellular fibrosis or steatohepatitis[40].

Concerning the F4 stage prediction, fourteen studies with 834 participants were analysed, using nine different cut-off values ranging from 7.15 to 34.9 kPa. The sensitivity of the TE varied from 75% to 100% and the specificity from 33% to 94%[6]. The most frequently used cut-off value for the prediction of cirrhosis in these studies was 12.5 kPa. Using this value for the prediction of cirrhosis in the meta-analysis yielded the following results: sensitivity 0.95 (95%CI: 0.87-0.98); specificity 0.71 (95%CI: 0.56-0.82); positive likelihood ratio (LR+) 3.3 (95%CI: 2.1-5.0); negative likelihood ratio (LR-) 0.07 (95%CI: 0.03-0.19)[6].

A comment is however required: due to the relatively small number of studies performed on patients with ALD, whose authors agreed to disclose the necessary data, the cited meta-analysis[6] could not establish the optimal cut-off values for the prediction of each fibrosis stage in ALD, which therefore still remains an open subject. The proposed cut-off values for the different stages of hepatic fibrosis may be used in clinical practice, but with caution, since those reported values were simply the most common cut-off values used by the study authors[6]; they are insufficiently validated and there is always the risk of overestimation of LS values in patients who are not abstinent from alcohol consumption[6,7].

The practical conclusion of this meta-analysis[6] is that TE may be used as a diagnostic method to rule out liver cirrhosis (F4) in people with ALD when the pre-test probability is about 51% (range 15% to 79%); TE may also help in ruling out severe fibrosis (F3 or worse). Liver biopsy remains an option if the identification of the stage of hepatic fibrosis or cirrhosis cannot be clearly made after a clinical follow-up or any other noninvasive test considered useful by the clinician[6].

WHAT IS THE SIGNIFICANCE OF LS IN PATIENTS WITH ALCOHOLIC LIVER DISEASES?

LS was proven to correlate well with the grade of fibrosis in various liver diseases[41]. However, the authors of the initial concept have admitted "it is unlikely that only one physical parameter (LS) can describe completely a complex biological system of

which fibrosis is just a part"[42]. Indeed, in a group of biopsied patients with hepatitis C virus infection, although fibrosis is the main predictor of LS, steatosis and necroinflammatory activity cannot be ignored as they could explain the stiffness variability within the same fibrosis stage. The relationship between LS and fibrosis (F), steatosis (S) and necroinflammatory activity (A) is illustrated in the equation[43]:

LS (logarithm) = 0.493 + (0.180 × F) + (0.034 × S) + (0.033 × A)

This is also true of ALD patients. However, the exact relationship between the 3 histopathological parameters and liver stifness still remains to be established in these patients.

Glisson's capsule, covering the liver, is distensible but not elastic. It follows that additional space-occupying tissue abnormalities, such as oedema and inflammation, cholestasis, congestion, cellular infiltrations, and deposition of amyloid may interfere with LS measurement, independently of fibrosis[44]; these confounding factors should be taken into account when interpreting the values of LS.

The necroinflammatory activity

The necroinflammatory activity influences LS, leading to an increase parallel with the histologic activity grade[14,45,46]. As a result, the tissue changes associated to an acute hepatitis may increase stiffness significantly, sometimes up to cirrhotic levels, due to cellular intumescence and sometimes to severe cholestasis[47]. The contribution of these non-fibrotic changes on stiffness was proven by the progressive decrease in stiffness alongside the decrease in transaminase levels[48,49]. On the other hand, in chronic hepatitis patients with transaminase flares, the increased stiffness is caused not only by pre-existing fibrosis but also by superimposed cellular intumescence[50]; consequently, the LS interpretation in patients with high ALT levels must be made with caution: at ALT levels above 2.5 × the normal limit, there is a risk to overestimate the fibrosis stage, which should be stated in the final examination result[19].

The influence of transaminase levels on the accuracy of fibrosis prediction by TE was highlighted by Mueller *et al*[34] in ALD patients, because AST levels > 100 U/L lead to an overestimation of fibrosis stage. The authors cautioned that active inflammation of the liver should first be excluded by blood tests, prior to the noninvasive assessment of fibrosis by TE. By excluding those patients with AST > 100 U/L at the time of LS assessment from this cohort, the area under the receiver operating characteristic (AUROC) for cirrhosis detection by FS improved from 0.921 to 0.945 while specificity increased from 80% to 90% at a sensitivity of 96%. A similar AUROC was obtained for fibrosis stages ⩾ F3 if LS measurements were restricted to patients with AST < 50 U/L. If transaminase levels are < 100 U/L, the LS value can identify liver fibrosis and can be used as a diagnostic tool[34].

Extrahepatic cholestasis

Extrahepatic cholestasis increases LS independently from fibrosis[51], and in patients requiring biliary drainage, the LS decreases with a mean of 1.2 ± 0.56 kPa for each 1 g/dL decrease in bilirubin. For this reason, it is recommended that before interpreting the LS results, a possible cholestasis be excluded through imaging and laboratory tests, in order to avoid the overestimation of fibrosis stage. The reasons underlying the high stiffness in cholestasis are unknown but could be related to tissue swelling, oedema and increased intracellular pressure due to impaired bile flow[44]. In addition, cholestasis may be a general phenomenon leading to higher LS in various chronic liver diseases, since intrahepatic cholestasis has been shown to correlate strongly with LS in patients with acute hepatitis[49] but also with ALD[34].

Congestive heart failure

Congestive heart failure may also lead to increased LS up to cirrhotic levels due to a higher content in hepatic blood, in up to 60% of patients[52-54]. In patients with decompensated congestive heart failure, LS is dramatically elevated and rapidly decreases during clinical recompensation due to diuretic therapy[55].

Liver infiltration, deposits, rare diseases

The rare infiltration with mast cells, also encountered sometimes in ALD patients, can also lead to dramatically increased LS[44]. An important noncancerous differential diagnosis of increased LS is amyloidosis[56,57].

Liver steatosis

The influence of steatosis on LS remains controversial. In some studies, steatosis did not significantly impact the stiffness, even after adjusting for fibrosis stage, but the proportion of patients with severe steatosis was too low to ensure the accurate quantification of any influence[7,17,45]. Other studies proved that for the same fibrosis stage and activity grade, the presence of steatosis lead to a significant increase in LS[43], while the morphometric analysis of the biopsy specimen proved that steatosis does indeed influence LS independently of fibrosis. This influence is negligible in cirrhosis but significant in non-cirrhotic patients[58]. Still, a steatotic, non-inflamed liver is usually softer, not stiffer. Further studies are therefore necessary to explain to what extent does steatosis influence LS values, especially in ALD patients.

LS AND ALCOHOLIC LIVER DISEASE FOLLOW-UP

Effect of detoxification on LS assessed by Fibroscan® in alcoholic patients

Gelsi *et al*[40] studied on a population from an addi-

ctology unit the changes in LS occurring after alcohol weaning over a period of 60 d, and compared these changes in relapser and abstinent patients. They found a rapid decrease in LS [-1.67% ± (-27.6%) on day 8] during detoxification in a high proportion of patients if abstinence was sustained: 41% of patients had lower values on day 8 and 66.7% on day 60. Relapsers were found to have a new increase in LS during follow-up after alcohol relapse.

Similar results were reported in a previous paper by Mueller *et al*[34] on 50 patients undergoing alcohol detoxification. The first finding was a parallel decrease in LS and AST values during alcohol detoxification. The second was that LS was more likely to decrease in patients with alcoholic liver disease with high initial AST levels, but remained stable once AST levels were below 100 U/L. The decrease in LS during alcohol detoxification could not be explained by changes in fibrosis stage given the short observation interval of 5.3 d. Therefore any change in LS must be attributed to other factors, most likely steatohepatitis[34].

Bardou-Jacquet *et al*[3] also confirmed these results during a much longer follow-up period (median 32.5 wk) with a precise control of the addiction. LS decreased after alcohol cessation over a long period of time, and this was of particular importance when the initial LS values varied between 8-16 kPa; these levels indicate significant fibrosis or cirrhosis in chronic hepatitis C, but should be interpreted with caution in ALD[3]. In this study, relapsers were found to have either an increase or a decrease in LS during follow up, possibly due to the level of alcohol consumption after relapse; relapsers could consume less alcohol during follow-up which could lead to a decrease in LS. This particular point should be assessed in a prospective study recording the precise alcohol amount consumed. If these results will be confirmed, then TE would have proven to be a useful tool in monitoring adherence during follow-up and fluctuations in alcohol consumption[3]. Considering that in this study LS and its variation were correlated with AST and GGT levels, the TE performance in estimating the fibrosis stage in ALD may be improved by the use of a coefficient based on liver enzyme values[3].

Prospective studies performed on large groups of biopsied patients followed up during alcohol withdrawal are, however, necessary and they must also establish the best interval between alcohol cessation and TE evaluation. Therefore, the interpretation of Fibroscan results in alcoholics must take into account whether alcohol consumption was continuous, the abstinence period as well as the biochemical tests at the moment of the examination (mainly AST, ALT and GGT).

Use of TE in monitoring disease progression in patients with alcoholic liver disease: Portal hypertension and hepatocellular carcinoma

In alcoholic cirrhosis, the evidence relating to the diagnostic accuracy of TE in relation to portal hypertension and oesophageal varices is weaker than that relating to fibrosis and cirrhosis[13].

Concerning portal hypertension, the studies performed on patients with various etiologies of liver cirrhosis report that TE can be quite effective in detecting patients with a high risk of having developed clinically significant elevations of hepatic venous pressure gradient (HVPG) or varices[41]. Several studies have shown that there is a good correlation between LS values and HVPG in patients with advanced liver diseases[37,59,60]. A recent meta-analysis found an excelent diagnostic performance of TE in predicting clinically significant PH (HVPG $\geqslant$ 10 mmHg) in patients with compensated chronic liver disease/cirrhosis, with an AUROC of 0.93[61]. While the correlation is excellent for HVPG values between 5 and 10-12 mmHg (typical of cirrhosis without evident clinical manifestations related to PH), it hardly reaches statistical significance for values above 12 mmHg[41,60].

Lemoine *et al*[38] analysed a group of 48 patients with alcoholic cirrhosis and 44 with viral C cirrhosis and found that, although all patients had compensated cirrhosis Child-Pugh class A, the LS was significantly higher in the former group (49.9 ± 21.7 kPa *vs* 25.7 ± 14 kPa, $P < 0.001$) and the area under ROC curve for the prediction of clinically significant portal hypertension was 0.94 ± 0.03; a cutoff value of 34.9 kPa had a sensitivity, specificity, PPV and NPV of 0.90, 0.88, 0.97 and 0.64, respectively, for the diagnosis of clinically significant portal hypertension. The cutoff values were different in the two studied groups, higher in the alcoholic cirrhosis group than in the viral C cirrhosis group (34.9 kPa *vs* 20.5 kPa), suggesting that LS values must be closely interpreted according to the cause of the liver disease[38]; apart from the amount and location of fibrosis, other elementary lesions such as steatosis and inflammation may also influence the LS values in alcoholic patients.

Even more uncertainty and controversy involves the possibility of predicting the presence and size of oesophageal varices (OV) based on LS values[41]. Some studies found a correlation between LS and the presence of oesophageal varices[60,62,63] with AUROCs ranging from 0.74 to 0.85 and cut-offs from 13.9 to 21.5 kPa. Although the sensitivity for the prediction of the presence of OV was high (76%-95%), specificity was in general not satisfactory (43%-78%).

A study by Nguyen-Khac *et al*[63] found that, in alcoholic cirrhosis, using a threshold of 47.2 kPa, FibroScan could predict the presence of large oesophageal varices with a sensitivity of 85% (95%CI: 67%-95%) and a specificity of 64% (95%CI: 53%-74%).

Some studies have highlighted the potential utility of spleen stiffness (SS) assessment for the prediction of the presence of OV and of the PH degree in cirrhotic patients[64,65]. Further validation is needed before the place of SS in clinical practice can be defined[41], especially in alcoholic liver disease.

Hepatocellular carcinoma

Several cross-sectional studies[66-69] identified that high LS values measured by TE are significantly associated with the risk of HCC. One of these studies was performed on a group including patients with alcoholic cirrhosis[69]. However, as mentioned in the EASL-ALEH guidelines on noninvasive tests for evaluation of liver disease severity and prognosis, these cross-sectional studies only describe the "static" phenomenon that patients with HCC have higher LS values than those without HCC, not considering the "dynamic" association between the progression or regression of liver fibrosis and the risk of future HCC development[41]. Several longitudinal prospective studies[70-80] have recently been published and stratified LS values were identified as an independent risk factor for HCC development. For example, in a study performed on patients with hepatitis C, compared with patients with LS values $\leqslant$ 10 kPa, those with higher LS values were at significantly higher risk of developing HCC (LS values, 10.1-15 kPa, HR = 16.7; LS values, 15.1-20 kPa, HR = 20.9; LS values, 20.1-25 kPa, HR = 25.6; and LS values, > 25 kPa, HR = 45.5)[68]. Nevertheless, few studies include ALD patients in their study groups[70,79], meaning that the cutoff values described in HCV and HBV patients cannot be extrapolated for ALD patients.

Concerning ALD patients, further studies are needed to expand the clinical prognostic usefulness of TE. In addition, optimal LS cut-off values to assess the risk of HCC development should be set up in the future in larger longitudinal prospective studies. Using TE to assess and monitor the risk of HCC development will help physicians to establish optimum treatment strategies. Further research should investigate whether the accuracy of the surveillance strategy can be enhanced by incorporating these noninvasive methods into the routine surveillance strategy[41].

NONINVASIVE ASSESSMENT OF STEATOSIS IN ALCOHOLIC LIVER DISEASE USING UNIDIMENSIONAL TE (FIBROSCAN®)

Steatosis is a frequent histological finding in patients with chronic liver diseases[81,82]. Ethanol consumption, the most popular cause for steatosis, induces fatty liver *via* multiple pathways[83]. An accurate method to detect and quantify steatosis would be extremely useful and it has been the subject of extensive research lately. One of the major obstacles in better defining the liver fat has been the lack of an easy, noninvasive and quantitative method to measure steatosis.

A novel noninvasive tool based on the evaluation of ultrasound attenuation using the Fibroscan® device (Echosens, Paris, France) has been developed, using a novel proprietary algorithm called controlled attenuation parameter (CAP)[84]. This parameter is an estimate of the total ultrasonic attenuation (go-and-return path) at the central frequency of the regular or M probe of the Fibroscan® (3.5 MHz) and is expressed in decibel per meter (dB/m). CAP is evaluated using the same radio-frequency data and the same region of interest as the region used to assess LS[85].

Since the development of this method, CAP has been used in some studies performed on patients with various diffuse liver diseases[84,86-94]. Among the histopathological parameters, these studies analyzed mainly the influence of steatosis and fibrosis and, in some studies, also that of necroinflammatory activity on CAP. One study, performed on NASH patients, included the influence of lobular inflammation and ballooning on CAP, apart from that of steatosis and fibrosis[95].

A recent study performed on a series of Romanian patients[96] has confirmed the preliminary results of previous studies[84,86-90,92-94] namely that, among all histopathological parameters assessed during various diffuse liver diseases, CAP is independently influenced only by the amount of steatosis, not by fibrosis, necroinflammatory activity, ballooning or lobular inflammation (quantified according to liver disease etiology). The CAP value increases alongside the increase in steatosis degree. Despite some overlap in adjacent steatosis grades, the overall differences between any two steatosis grades are statistically significant, except between $\geqslant$ 34%-66% and 67%-100% fatty load, which was also reported by several authors[87-89,97,98]. Moreover, this situation is also encountered when quantifying steatosis using ^{1}H-magnetic resonance spectroscopy[98], which raises the suspicion of bias in steatosis quantification for those grades on liver biopsy[96].

In a meta-analysis assessing the CAP accuracy for steatosis detection[99], the median optimal CAP cut-off values were 232.5 dB/m, 255 dB/m and 290 dB/m for steatosis involving $\geqslant$ 11%-33% (S1), $\geqslant$ 34%-66% (S2) and 67%-100% of hepatocytes (S3), respectively, and the summarized sensitivity and specificity values were 0.78 (95%CI: 0.69-0.84) and 0.79 (95%CI: 0.68-0.86) for $\geqslant$ S1, 0.85 (95%CI: 0.74-0.92) and 0.79 (95%CI: 0.71-0.85) for $\geqslant$ S2, and 0.83 (95%CI: 0.76-0.89) and 0.79 (95%CI: 0.68-0.87) for S3.

Few ALD patients have been included in studies performed so far, evaluating the utility of CAP in assessing steatosis in various difuse liver diseases; for this reason, a complete analysis of patients with this etiology was never acomplished. Certain aspects of this analysis still remain to be clarified in future studies in alcoholic patients[44,100]: Which are the CAP cutoff values for the prediction of steatosis grade in ALD? To what extent does the histology of liver steatosis (micro- or macrovesicular) influence CAP? Is there a quantitative relationship between the location and histological type of the hepatitis, the transaminase level and LS? What is the diagnostic value of LS in more complex clinical settings, for example a patient

with combined alcoholic liver fibrosis, steatohepatitis, and cardiomyopathy? How does CAP change in response to fast kinetics such as alcohol detoxification, binge drinking, after meals and the intake of certain drugs?

CONCLUSION

In conclusion, in alcoholic liver disease, unidimensional TE is useful mainly in 2 areas: to identify patients with fibrosis, so that efforts may be made to prevent the development of cirrhosis, and to identify patients with cirrhosis, enabling a better monitorization for the development of complications such as oesophageal varices and HCC. The results may be influenced by factors other than the degree of fibrosis present in the liver, mainly acute alcoholic hepatitis. The current drinking status is also relevant. Prospective studies performed on large groups of biopsied patients are, however, necessary, to establish the optimal cut-off values of LS and CAP for the prediction of each fibrosis and steatosis grade.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis B Virus

Hepatitis B among Asian Americans: Prevalence, progress, and prospects for control

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Abstract

After tobacco use, chronic hepatitis B (CHB) viral infections are the most important cause of cancer globally in that 1 out of 3 individuals have been infected with the hepatitis B virus (HBV). Though infection rates are low (< 1%) in the United States, Asian Americans who comprise about 6% of the population experience about 60% of the CHB burden. This paper reviews the magnitude of hepatitis B (HBV) burden among Asian Americans and the progress being made to mitigate this burden, primarily through localized, community-based efforts to increase screening and vaccination among Asian American children, adolescents, and adults. This review brings to light that despite the numerous community-based screening efforts, a vast majority of Asian Americans have not been screened and that vaccination efforts, particularly for adults, are sub-optimal. Greater efforts to integrate screenings by providers within existing healthcare systems are urged. Evidence-based strategies are offered to implement CDC's three major recommendations to control and prevent hepatitis B through targeted screening and enhanced vaccination efforts.

Key words: Hepatitis B; Asian Americans; Chronic hepatitis B; Vaccination

Core tip: Hepatitis B viral infections disproportionately affect Asian Americans. Untreated, hepatitis B viral infections can lead to hepatocellular carcinoma that is almost universally fatal. Unfortunately, a vast majority of Asian Americans have not been screened. To reduce the HBV burden, screening both in community and clinical settings must be accelerated; and both physicians and patients must see the need for testing. Based on test results, those who screen positive must be referred to appropriate care and those without

natural immunity, recommended for vaccination.

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INTRODUCTION

Worldwide, two billion people (1 in 3) have been infected with the hepatitis B virus (HBV), making HBV one of the most common and serious infections in the world[1]. Based on this magnitude, the HBV, in its chronic form leading to hepatocellular carcinoma (HCC), are the most important cause of cancer globally after tobacco use[2]. Unless detected and treated early, HBV-induced HCC is a highly fatal form of primary liver cancer[3-6]. More than 75% of liver cancers are attributed to HBV infections[7,8], and of the 40 million individuals chronically infected with HBV, unless medical intervention occurs, 15% to 25% will progress to HCC[9]. Although the overall prevalence of chronic HBV (CHB) infection is low in the United States (< 1%), increasing immigration from hepatitis B endemic areas (where the hepatitis B surface antigen prevalence is ⩾ 2%), such as East Asia, Pacific Islands, and parts of Africa and Eastern Europe[10], have led to rising health disparities among these groups, with immigrants now having the same risk of CHB as their country of birth[5,11,12].

In the United States, Americans of Asian ancestry (otherwise known as Asian Americans), comprise less than 6% of the United States population, however, represent the highest and most disproportionate (approximately 58%)[6], burden for HBV-linked HCC[6,13-17]. Per 100000, Asian Americans experience the highest incidence for cancers of the liver and intrahepatic bile (male: 21.2 *vs* 8.9 for Whites; female: 8.0 *vs* 3.0 White) and mortality rates (male: 14.5 *vs* 7.3 for Whites; female: 6.0 *vs* 3.0 for Whites)[17]. Most dramatically, the hepatitis B seroprevalence rate among foreign-born Asian/Pacific Islander women of childbearing ages was 8.9% compared to 0.08% for non-Hispanic White mothers for a disparity rate of 110:1[18]. Since 2000, Asian Americans experienced a 51% increase in population, the highest growth rate of any racial/ethnic group[19]. By the year 2050 there will be 33.4 million Asian Americans living in the country representing a 213% population increase compared to a 49% increase for the rest of the Nation[20]. At the same time, the highest increases in liver cancer cases in the United States are expected among Hispanics, Asians, and Pacific Islanders[21]. Thus, addressing liver cancer is a very relevant and significant arena for impacting cancer health disparities with great importance for the United States population. To set the context for this paper, we first present important and relevant characteristics of Asian American populations based on the United States Census data.

According to the Office of Budget Management, "Asian" refers to "a person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent, including, for example, Cambodia, China, India, Japan, South Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam"[22]. This categorization represents at least 32 subgroups and over 100 spoken languages and dialects[23]. Asian Americans are the fastest growing racial group (between 2000 and 2010 the Asian populations experienced a 46% increase) and it is estimated that in 2013, 19.4 million Asian Americans resided in the United States[24]. In terms of demographics, Asian Americans are the only racial population in the United States with a bimodal distribution for major demographic risk factors related to health outcomes: education, income, insurance status, and age[25]. While the median household income for all Asian Americans is $72472; across ethnic groups, the median income ranges from $48249 for Hmong Americans to $98562 for Asian Indians. The median age for Asian Americans is 36.3 and that ranges from 22.2 years of age for Hmong Americans to 49.1 years of age for Japanese Americans. The poverty rate for the Asian alone category is 12.7%, and is as low as 8% for Japanese Americans to as high as 26.3% for Hmong Americans. As an aggregate, 14.6% of Asian Americans do not have health insurance, with 24.2% of Korean Americans and 6.9% of Japanese Americans living without coverage. In terms of education, 86.2% of Asian Americans have their high school diploma (compared to 86.4% of the United States population); however that number ranges from 64.8% for Cambodian Americans to 95.1% for Japanese Americans[26]. These stark and alarming differences between the Asian groups are masked by the aggregated data; for Asian Americans these differences create devastating disparities in cancer outcome. Asian Americans have one of the lowest cancer screening rates[27] and are the least represented ethnic population in cancer control research studies and targeted intervention programs by the United States Federal government[28].

LITERATURE RESEARCH

The objectives of this review are to: (1) focus on the six Asian American populations with the largest numbers who may be chronically infected with HBV (see Table 1 below)[6], *i.e.*, those of Cambodian, Chinese, Filipino, Hmong/Laotian, Korean, Vietnamese ancestry and the limitations of these data based on PubMed papers published since 2000; (2) highlight the progress to reduce the burden of HBV among Asian Americans;

Table 1 Chronic hepatitis B prevalence rate of foreign-born with chronic hepatitis B living in the United States for selected Asian Countries[6]

Country	Chronic hepatitis B prevalence rate (%)
Laos (home to the Hmong)	13.61
Vietnam	12.48
China	12.25
Cambodia	10.27
Philippines	7.36
Thailand (home to the Hmong)	5.97
South Korea	5.26

and (3) recommend public health-oriented measures for mitigating the HBV burden among Asian Americans. For the first objective, we conducted PubMed searches in April 2015 for "Hepatitis B prevalence among Asian Americans" supplemented by papers that the authors were aware of. For the second objective, we chose to focus on progress exemplified by: (1) Federally-funded, randomized controlled, community-based interventions to increase HBV screenings and those listed on ClinicalTrials.gov; (2) results of increasing HBV vaccination efforts in PubMed-retrieved publications from 2001-April 2015; and (3) linkage to care for HBV-infected Asian Americans. For (2) and (3) we conducted PubMed searches for "Increasing HBV vaccinations among Asian Americans" and "Linkage to care for HBV positive Asian Americans", and supplemented the results with articles that the authors were aware of. The two activities of increasing HBV vaccinations and linkage to care for HBV positive patients are in accordance with the latest American Association for the Study of Liver Diseases guidelines for HBV[28,29] and the CDC's recommendations[10,30,31]. In addition, increasing serological testing for HBV aligns with the 2014 United States Preventive Services Task Force grade "B" guidelines[32]. The decision of limiting this review to Federally-funded, randomized controlled, community-based interventions to increase HBV screenings and those listed in ClinicalTrials.gov was based on the premise that randomized, controlled trials represent greater scientific rigor than one-time health fair-type screenings or uncontrolled or quasi-experimentally conducted interventions. Likewise, we limited our focus on progress for documenting increased HBV vaccinations and linkage to care for HBV positive Asian Americans to PubMed retrievable peer-reviewed papers. For the third objective, we used the Centers for Disease Control and Prevention's Recommendations for Public Health Management of Persons with Chronic HBV infection and Strategy for the Elimination of Hepatitis B Transmission[10] and the recommendations of the Advisory Committee on Immunization Practices[30,31] as the framework for compiling empirically-derived findings that have demonstrated impact on reducing the HBV burden.

RESULTS

HBV prevalence among Asian Americans

The National Health and Nutrition Examination Survey (NHANES), the only source of nationally representative hepatitis B seroprevalence data for the United States[33], has acknowledged limitations of under-representation of Asian Americans and the lack of nativity data in their survey which are critical to identify at-risk populations[11]. At press time, the report from the 2011-12 NHANES survey that intentionally over-sampled Asian Americans had not been released**.** Meanwhile, to address these data deficiencies, various investigators have taken different approaches to determine HBV prevalence among Asian Americans on a national and community basis.

On a national basis, Kowdley *et al*[6] used CHB rates from countries of origin and United States Census data of distinct population groups by national origin to determine rates. Their findings indicated that of the 1.32 million people living with CHB, 58% (about 765000 individuals) migrated from Asia. Similarly, on a national basis, Mitchell *et al*[5] estimated that during 1974-2008 there were about 1.3 million new cases of chronic HBV infection imported into the United States. The largest number of imported CHB cases came from the Philippines, China, and Vietnam, who together accounted for 37% of the total imported CHB cases during this period. The only published composite report based on accumulating data from 31 community-based efforts in various parts of the United States indicated that over 21817 people have been screened annually with an average hepatitis B surface antigen rate of 8.1%[34].

At the community level, the numbers of publications that report on HBV screenings in PubMed are now in the triple digits and is increasing monthly. By using the search term, "hepatitis B AND Asian Americans", 175 articles were identified in April 2015. These screenings were open to all and hence reflect convenience samples resulting in varying numbers of individuals screened for HBV and with HBV positivity rates reported either by ethnic group or as an aggregated Asian American group. Typically more female than male participated and there were generally higher HBV positivity rates among males. Despite limitations in generalizability by ethnicity, in later funded studies, CDC concluded that the 9 community sites could appropriately identify CHB individuals from high HBV prevalence populations and refer them to care; they highlighted the findings from three of those sites[35]. Reflecting on the composite 6.6% hepatitis B surface antigen (HBsAg) positivity rate for those three sites, our own "Thousand Asian American Study" (that was not among the three reported in the MMWR article) resulted in a similar overall HBV positivity rate of 6.5%.

However, by dis-aggregation by ethnicity and gender, Hmong and Vietnamese men had considerably higher rates, 14.3% and 13.6% respectively, signifying the importance of interpreting dis-aggregated rates[36]. Having dis-aggregated data by ethnicity and gender allows us to target our screening and HBV prevention and control efforts more precisely as these efforts require linguistically appropriate and culturally-specific approaches.

Progress being made

In addition to the localized HBV prevalence rates gathered through community-based efforts, NIH-funded investigators have elucidated theoretically-based and culturally appropriate interventions that have empirically guided measures to promote hepatitis B testing in Cambodian, Hmong, Korean, and Vietnamese[37-40]. These investigators offer principles that have guided community-centered screening interventions.

From the three NIH-funded randomized controlled community-centered interventions that have been published, we documented that bilingual/bicultural Cambodian ($P < 0.001$)[41], Hmong ($P < 0.0056$)[42], and Korean ($P < 0.001$) lay health educators[43] are effective in increasing HBV serological testing rates in community settings, however, the physician's role in influencing serological testing within the context of a healthcare system appears to be far more efficient and desired[42,44]. Undoubtedly, community-based HBV screenings reach many who might not otherwise be reached; however, validating receipt of HBV screening results and follow up of care can be challenging due to a lack of health insurance and the administrative burden of reviewing paper medical charts from a mosaic of healthcare providers[42,43,45]. This is in contrast to the efficiency and effectiveness of electronic health records as a means of identifying those needing to be tested and follow-up of test results which also achieved statistically significant screening and testing in a randomized, controlled study[46]. Leveraging electronic medical records systems to determine whether HBsAg have been completed coupled with an algorithm that selects for typical Asian surnames as surrogate markers of nativity in intermediate to high HBsAg regions represent perhaps more efficient means of targeted screening. However both approaches: "out-reach" (clinical) through community-centered efforts and "in-reach" (nonclinical) are needed[47].

Among the more sophisticated and controlled studies to evaluate screening interventions are studies registered on the National Library of Medicine operated website, ClinicalTrials.gov: *e.g.*, "Community-based Hepatitis B interventions for Hmong adults" and "Increasing Hepatitis B screening among Korean church attendees" that have reported their results[42,43]. The third study, "Patient-Centered Care and Asian Americans" is currently in progress and through a randomized, controlled trial will evaluate a mobile app plus a physician panel notification (intervention) compared to a physician panel notification only (control), in increasing hepatitis B and C testing among Asian Americans[48].

Information on HBV vaccination efforts among Asian Americans in PubMed retrieved literature is sparse. Out of the 7 articles retrieved using the search term, "Increasing HBV vaccinations for Asian Americans", none reported actual numbers of vaccinations but rather referred to vaccinations in the context of preventing HBV infections. Substantial progress due to vaccinations can be inferred through the 68% decline in HBV infection prevalence among United States children (which includes both United States born[49] and foreign-born Asian children). Among United States adolescents as a whole, the HBV vaccination rate is at 93.2% but is slightly lower among Asian teens (87.8%)[50] and so there is room for improvement. However, the greatest need is the high CHB rates among adults[36]. Screening efforts were sub-optimal (under 50%) even among Asian primary care providers who realize the significance of HBV and vaccination levels for their adult Asian patients[51].

Thus, progress in preventing and controlling HBV among must transcend screening to HBV vaccinations where appropriate and linkage to care as needed. Among the CDC-funded screening to linkage to care grantees, referral to care ranged from 56%[47] to 86%[35] to 92%[36]. These percentages exceed the estimated national baseline of about 40%[4] or approximately 33% among Racial and Ethnic Approaches to Community Health communities[52]. More work will be needed in this arena.

DISCUSSION

In the United States, deaths due to cancers of the liver and intrahepatic bile duct lead all organ sites in terms of annual percent change (3.6% for males and 2.9% for females) while deaths for cancers as a whole dropped by 1.4% and 0.2% for males and females respectively. At the same time, cancers of the liver and intrahepatic bile duct also experienced a considerably lower five-year survival rates (18%) compared to all sites (68%)[17]. Liver cancer mortality rates lead all other organ sites in the cumulative increases in cancer deaths (50.28% among men and 28.83% among women)[13]. Liver cancer mortality rates have continued to increase with age in all racial/ethnic groups between 2006 to 2010[14]. While multiple conditions contribute to these increases in mortality including Hepatitis C viral infections[53], high body mass index[54], diabetes[55], liver cancer, and in particular, CHB for Asian Americans[56], HCC is a cancer with an extremely dismal survival rate, especially for Asian Americans[29,57]. Thus, the importance of focusing on mitigating the HBV-HCC burden among Asian

Table 2 Recommendations to mitigate the hepatitis B virus burden among Asian Americans

CDC's recommendations	Evidence-based strategies
1.Screen persons born in areas with ≥ 2% hepatitis B surface antigen (HBsAg) rates[10]	a.Ask persons which country they were born b.Encourage providers to recommend HBsAg testing for their at-risk Asian American patients c.Educate and encourage patients to ask their providers whether they should be tested for HBsAg[44] d.Use typical Asian names from EHRs to determine if they have been tested for HBsAg and then to screen them at the next opportunity[46] e.Collaborate with Asian American-serving organizations to hold screening events[36]
2.Vaccinate Asian American infants and children[30]	a.Verify that hospitals and birthing facilities are providing birth dose vaccinations b.If not vaccinated at birth, vaccinate. Note that in many states, verification of hepatitis B virus (HBV) vaccinations may be a requisite for school enrollment
3.Vaccinate Asian American adults. Note: serologically test for HBsAg first and after test results are known, determine if vaccination is appropriate[31]	a.Consider follow up vaccination programs for adults after serological testing for HBV for those who need vaccination[36]

Americans cannot be discounted while the under-resourced efforts cannot be denied[58].

Mitigating the progression to HCC through earlier detection and preventing CHB infections and appropriate medical management of CHB for infected individuals is the recommended pathway to eliminating these disparities and carries with it the possibility of being a health disparity that could be eliminated[59,60]. The 2014 designation by the United States Preventive Services Task Force of a grade of "B"[32], plus the evidence for the cost-effectiveness of screening, particularly in outpatient settings[29,57] are factors that support HBV screening and referral to treatment[61,62]. Unfortunately, because CHB is typically asymptomatic, testing is infrequent and 65% of infected Americans are unaware of their HBV status[30,31]. Even in the Chronic Hepatitis Cohort Study of 1.2 million people with access to care, only 18.8% were tested for HBV[32]; our results were similar, 17.3%-18.5%[46]. In addition, of those diagnosed with CHB, only about 40% are referred and linked to care[4], an inadequate number of providers are trained in appropriate care, and few patients are referred to appropriate care[12,63,64].

CONCLUSION

After tobacco use, chronic hepatitis B viral infections are the most important cause of cancer in the world. While Asian Americans only comprise 6% of the United States population, they bear almost 60% of that burden and the disparity rate is 110:1 for the HBV seroprevalence rate among foreign-born Asian American women of childbearing ages compared to non-Hispanic White mothers. Based on calculations of CHB rates from countries of origin and United States census data, the numbers of Americans of Asian ancestry with chronic hepatitis B is approaching a million. Unfortunately, less than 50% of at-risk Asian Americans know their HBV status and/or have been serologically tested and while the vaccination rates for Asian American children and adolescents are higher, vaccinations for Asian American adults are sub-optimal.

Based on this review, the authors suggest the following strategies that align with the three principal CDC recommendations as they relate to mitigating the HBV burden among Asian Americans (Table 2).

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis B virus

Role of occult hepatitis B virus infection in chronic hepatitis C

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Abstract

The development of sensitive assays to detect small amounts of hepatitis B virus (HBV) DNA has favored the identification of occult hepatitis B infection (OBI), a virological condition characterized by a low level of HBV replication with detectable levels of HBV DNA in liver tissue but an absence of detectable surface antigen of HBV (HBsAg) in serum. The gold standard to diagnose OBI is the detection of HBV DNA in the hepatocytes by highly sensitive and specific techniques, a diagnostic procedure requiring liver tissue to be tested and the use of non-standardized non-commercially available techniques. Consequently, in everyday clinical practice, the detection of anti-hepatitis B core antibody (anti-HBc) in serum of HBsAg-negative subjects is used as a surrogate marker to identify patients with OBI. In patients with chronic hepatitis C (CHC), OBI has been identified in nearly one-third of these cases. Considerable data suggest that OBI favors the increase of liver damage and the development of hepatocellular carcinoma (HCC) in patients with CHC. The data from other studies, however, indicate no influence of OBI on the natural history of CHC, particularly regarding the risk of developing HCC.

Key words: Occult hepatitis B virus infection; Silent hepatitis B virus infection; anti-hepatitis B core antibody; Hepatitis B virus infection; Cirrhosis; Hepatocellular carcinoma

Core tip: Occult hepatitis B infection (OBI) is a virological condition characterized by a low level of

hepatitis B virus (HBV) replication with HBV DNA detectable in liver tissue in the absence of detectable surface antigen of HBV in serum. Some studies indicate that OBI may favor the increase of liver fibrosis and the development of hepatocellular carcinoma in patients with chronic hepatitis C, whereas other investigations refute this. Here, we review all the available data on this topic and discuss the possible influence of OBI on the natural course of chronic hepatitis C.

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INTRODUCTION

Approximately 170 million individuals are chronically infected with hepatitis C virus (HCV) worldwide[1-4]. HCV is a small, enveloped, positive-sense, single-stranded RNA virus of the genus *Hepacivirus* of the *Flaviviridae* family. Phylogenetic analysis of HCV isolates has generated the viral classification into six major genotypes (from 1 to 6) and more than 100 subtypes[5,6]. HCV is transmitted by percutaneous exposure to infected blood through intravenous drug injection and invasive medical procedures, and by permucosal exposure through unprotected intercourse with multiple partners[7,8], particularly in human immunodeficiency virus (HIV)-positive men who have sex with men[9-12].

HCV causes acute hepatitis that is frequently asymptomatic, and in its symptomatic form, it is characterized by nausea, malaise, and jaundice. The acute HCV infection resolves spontaneously in about one-third of the cases[13,14], whereas the remaining two-thirds remain infected, circulate anti-HCV and HCV RNA, and usually show an indolent course or a slow progression to liver cirrhosis and hepatocellular carcinoma (HCC)[15]. In some cases, however, spontaneous acute exacerbations may develop, characterized by one or more peaks of the aminotransferase serum levels above the previous values[16-22], which can frequently induce a deterioration of the liver disease. In some cases the progression to liver cirrhosis and HCC is rapid[15], particularly when co-morbidities, an unfavorable genetic background, and unsafe lifestyle factors are present. Indeed, the outcome of chronic hepatitis C (CHC) is influenced by associated host factors (sex, age at infection, routes of transmission, immune response, genetic background), viral factors (HCV genotype and viral quasispecies), co-morbidities (viral co-infection, insulin-resistance, liver steatosis, immunosuppressive clinical condition) and lifestyle factors (alcohol intake)[23-30].

The development of sensitive assays to detect small amounts of hepatitis B virus (HBV) DNA has favored the identification of occult HBV infection (OBI), a virological condition characterized by a low level of HBV replication with HBV DNA detectable in the liver cells in the absence of detectable surface antigen of HBV (HBsAg) in serum. In patients with CHC, OBI has been identified in about one-third of HBsAg-negative/anti-HCV-positive subjects in the Mediterranean Basin and in more than 50% in East Asian countries[31-36]. Considerable data suggest that in patients with CHC, OBI may contribute to chronic liver damage and to the development of HCC[24,31,37-40]. Other studies, however, indicate that OBI does not influence the natural history of HCV infection, particularly as regards the risk of HCC development[41-43]. In this review article, which takes into account all the available literature data, the possible role of OBI in modifying the clinical course of CHC is evaluated and discussed.

DEFINITION OF OBI

OBI has been defined as the presence of viral DNA in the liver tissue (regardless of HBV DNA detectability in serum) of individuals testing negative for serum HBsAg[36]. The gold standard to diagnose OBI is the detection of HBV DNA in the hepatocytes by highly sensitive and specific techniques [real-time polymerase chain reaction (PCR), nested PCR, and the use of oligonucleotide primers specific for different HBV genomic regions], a diagnostic procedure requiring liver tissue to be tested and the use of non-standardized non-commercially available techniques. Consequently, in everyday clinical practice, the detection of anti-hepatitis B core antibody (anti-HBc) in serum of HBsAg-negative subjects, a sign of previous acute hepatitis B (AHB), is used as a surrogate serum marker to identify subjects with OBI[39,43-49]. This option is supported by the observation that in patients experiencing immunosuppression, OBI, as defined by the presence of HBV DNA in liver tissue, mostly occurs in HBsAg-negative/anti-HBc-positive patients[44,50,51]. The data from a previous investigation on 89 HBsAg-negative patients with CHC showed the presence of HBV DNA in plasma, peripheral blood mononuclear cells, and/or liver tissue in 60% of the anti-HBs/anti-HBc-positive patients, in 80% of the anti-HBs-negative/anti-HBc-positive patients, and in 10% of those lacking both antibodies[44].

MECHANISMS OF LIVER DAMAGE BY OBI

In some cases, an underhand activity of the HBV genome is the persistence of mild hepatocellular necrosis for years after the resolution of self-limiting AHB[52,53]. The mechanism of liver damage due to OBI is still unclear, but there is some evidence that viral

factors may play a role in its development and in the related liver damage. In fact, the persistent synthesis of minute undetectable amounts of the virus or other viral transcripts produced by the HBV covalently closed circular DNA (cccDNA) seems capable of maintaining the HBV-specific memory T-cell response[33,54] and the production of cytokines, such as tumor necrosis factor-α and interferon-γ[55,56]. In addition, mutations in the X region of HBV may reduce the ability of the X protein to transactivate host cellular proteins essential for viral replication, which may lead to the reduction of HBV DNA replication and the lack of HBsAg serum expression[57].

We should remember, however, that a rare escape mutation in the S region decreases the reactivity in the HBsAg detection assays[58] and is responsible for an "overt" HBsAg-negative infection that might mimic OBI.

OBI AND THE PROGRESSION OF LIVER FIBROSIS

The impact of OBI, as detected by the presence of serum anti-HBc, on the progression of liver fibrosis in patients with CHC

As mentioned above, the detection of serum anti-HBc has been used as a surrogate marker of the presence of liver HBV DNA to detect OBI in numerous investigations exploring the correlation between this virological condition and liver fibrosis in patients with CHC (Table 1). In the year 2000, our group[24] published a cross-sectional, case-control study on 174 Caucasian HBsAg-negative CHC patients. We showed that the prevalence of cases with cirrhosis in the anti-HBc-positive subgroup was significantly higher than in the anti-HBc-negative subgroup, suggesting a role of OBI in fibrosis progression. Similar data were obtained in a cross-sectional study performed in the same period by De Maria *et al*[37] on 285 HCV-infected patients. A few years later, a cross-sectional study[38] confirmed the relationship between the presence of anti-HBc and liver cirrhosis in 119 Italian anti-HCV-positive/HBsAg-negative patients. A study on 129 Portuguese anti-HCV-positive patients published in 2005 found an independent association between previous HBV infection and biopsy-proven liver cirrhosis[59]. Subsequent studies further confirmed the unfavorable influence of OBI, as detected by the presence of anti-HBc in HBsAg-negative patients, on the clinical course of CHC. A cross-sectional Brazilian study[60] found OBI was a predictor of significant necroinflammation and fibrosis; El-Sherif *et al*[61] demonstrated that the prevalence of cases with advanced liver disease was higher in patients with OBI than in those without. Coppola *et al*[62] demonstrated that OBI was an independent predictor of HCV-related cirrhosis in a cross-sectional study on 222 patients from southern Italy.

Conflicting data have been published by other authors. Verbaan *et al*[63] did not find any association between OBI and the progression to cirrhosis in 99 CHC patients, whereas in an Egyptian study, patients with OBI were more likely than those without to show HCV-related cirrhosis[64]. No association was observed between serum anti-HBc and the degree of liver fibrosis in a study from Spain[65] or between anti-HBc and the entity of necroinflammation or fibrosis in a French study[66].

The impact of OBI, as detected by the presence of HBV DNA in liver tissue, plasma, or peripheral blood mononuclear cells, on the progression of liver fibrosis in patients with CHC

Table 2 shows the data from several studies on the relationship between the degree of liver fibrosis and the presence of OBI, as demonstrated by detecting HBV DNA in the liver tissue, plasma, or peripheral blood mononuclear cells (PBMC) of HBsAg-negative patients with CHC (Table 2). One of the first studies to suggest a clinical impact of OBI was a cross-sectional study published in 1999 by Cacciola *et al*[31], which showed in 200 CHC patients that 33.3% of those with detectable liver HBV DNA had cirrhosis whereas only 19.4% of HBV-DNA-negative patients did. Similar data were observed in 203 HCV-infected patients in a French study published in 2007[67], in which patients with plasma HBV-DNA showed more advanced fibrosis ($P < 0.001$) than those who were HBV-DNA-negative. In 2008, Matsuoka *et al*[68] tested 468 Japanese HBsAg-negative patients with CHC for the presence of plasma HBV DNA and found over a mean follow-up period of 6.7 years that cirrhosis and HCC occurred more frequently in those with OBI than in those without. These data were confirmed in a prospective study from Italy, in which HBV DNA was detected in the liver tissue of 326 CHC patients[69], and progression to cirrhosis or development of HCC were more frequent in those with OBI than in those without.

Conflicting data, however, have come from several studies, with all but one detecting HBV DNA only in plasma. In a Japanese study on 65 HCV-infected patients, liver cirrhosis was detected with a similar frequency in CHC patents with or without OBI[35]. In 2004, Toberson *et al*[70] reported no association between OBI and the grading or staging in 180 anti-HCV-positive drug users. A cross-sectional study published in the same year on 59 Brazilian patients[71] showed a similar degree of liver fibrosis in those with or without OBI. Hui *et al*[72,73] published in 2006 two retrospective cohort studies on 74 CHC patients and 118 subjects with a recurrent HCV infection after liver transplantation, respectively. In both studies, liver fibrosis, as detected by comparing two consecutive liver biopsies, showed a similar increase in patients with or without OBI. In addition, Sagnelli *et al*[44] did not find any association between the degree of liver

Table 1 The studies evaluating the role of anti-HBc in the development of cirrhosis in HBsAg-negative patients with chronic hepatitis C

First author, year	No. of patients	Country	Type of study	Cirrhosis, positive/tested, *n/n* (%)		*P* value
				HBcAb$^+$	HBcAb$^-$	
Verbaan 1998[63]	99	Sweden	Cross-sectional	10/44 (22.7)	10/55 (18.2)	NS
De Maria 2000[37]	285	United States	Cross-sectional	29/90 (32.2)	41/195 (21.0)	< 0.05
Sagnelli 2000[24]	174	Italy	Case-control	9/76 (11.8)	6/98 (6.1)	< 0.005
Giannini 2003[38]	119	Italy	Cross-sectional	20/48 (41.6)	15/71 (21.0)	0.020
Dinis-Ribeiro 2005[59]	129	Portugal	Cross-sectional	14/30 (46.6)	32/99 (32.3)	HR:1.35 (1.01-2.69)[1]
Helmy 2006[64]	169	Saudi Arabia	Cross-sectional	14/85 (16.5)	45/84 (53.6)	0.0001
Laguno 2008[65]	238	Spain	Cross-sectional	78/142 (55)[2]	49/96 (51)[2]	0.720
Carvalho-Filho 2009[60]	111	Brazil	Cross-sectional	24/31 (77.4)[3]	40/80 (50.0)[3]	0.017
El-Sherif 2009[61]	100	Egypt	Cross-sectional	68/71 (95.8)	23/29 (79.3)	0.009
Levast 2010[66]	140	France	Cross-sectional	5/45 (11.1)	16/95 (16.8)	NS
Coppola 2014[62]	222	Italy	Cross-sectional	21/77 (27.3)	12/145 (8.3)	< 0.009

[1]Hazard ratio for progression to cirrhosis in HBsAb/HBcAb$^+$ patients; [2]Advanced fibrosis (Scheuer score > 2); [3]Advanced fibrosis (Metavir score F2-F4). NS: Not significant.

Table 2 The studies evaluating the role of hepatitis B virus DNA in serum and/or liver tissue in the development of cirrhosis in surface antigen of hepatitis B virus-negative patients with chronic hepatitis C

First author, year	No. of patients	Country	Type of study	Liver disease	Sample for HBV-DNA detection	Cirrhosis, positive/tested, *n/n* (%)		*P* value
						OBI$^+$	OBI$^-$	
Cacciola 1999[31]	200	Italy	Cross-sectional	CH/cirrhosis	Liver	22/66 (33.3)	26/134 (19.4)	0.040
Fukuda 1999[35]	65	Japan	Cross-sectional	CH/cirrhosis	Serum	5/34 (14.7)	3/31 (9.7)	NS
Giannini 2003[38]	119	Italy	Cross-sectional	CH/cirrhosis	Serum	2/8 (25.0)	32/111 (28.8)	NS
Silva 2004[71]	59	Brazil	Cross-sectional	CH/cirrhosis	Serum	2/10 (20.0)	6/49 (12.0)	NS
Toberson 2004[70]	180	United States	Cross-sectional	CH/cirrhosis	Serum	8/81 (9.9)[1]	11/99 (11.1)[1]	NS
Hui 2006[72]	74	United States	Cohort	CH/cirrhosis	Serum	6/31 (19.4)[2]	8/43 (18.6)[2]	NS
Hui 2006[73]	118	United States	Cohort	Liver transplantation	Serum	8/41 (19.5)[2]	13/77 (16.9)[2]	NS
Mrani 2007[67]	203	France	Cross-sectional	CH/cirrhosis	Serum	28/47 (60.0)	52/156 (33.3)	< 0.001
Laguno 2008[65]	90	Spain	Cross-sectional	CH/cirrhosis	Serum	8/15 (53.3)[3]	37/75 (49.3)[3]	NS
Matsuoka 2008[68]	468	Japan	Cross-sectional	CH/cirrhosis	Serum	37/204 (18.1)	28/264 (10.6)	0.002
Sagnelli 2008[44]	89	Italy	Cohort	CH/cirrhosis	Serum/PBMC/liver	10/37 (27.0)[3]	19/52 (36.5)[3]	NS
Emara 2010[74]	155	Egypt	Cross-sectional	CH/cirrhosis	Serum	0/6 (0.0)	4/149 (2.7)	0.020
Squadrito 2013[69]	326	Italy	Cohort	CH/cirrhosis	Liver	30/128 (23.4)	25/198 (12.6)	< 0.01

[1]Advanced fibrosis (Ishak score 3-6); [2]Severe fibrosis (Metavir score F3-F4); [3]Advanced fibrosis (Scheuer score > 2); [4]Severe fibrosis (Scheuer score 3-4). CH: Chronic hepatitis; PBMC: Peripheral blood mononuclear cells; NS: Not significant; HBV: Hepatitis B virus; OBI: Occult HBV infection.

fibrosis and OBI in a prospective study where OBI was assessed through the detection of HBV DNA in plasma, PBMC, and liver tissue in 89 patients with CHC. Finally, Emara *et al*[74] studied 155 Egyptian CHC patients and found the prevalence of cases with cirrhosis in patients with circulating HBV DNA was significantly lower than in those without.

OBI AND THE OCCURRENCE OF HCC

There is biological, epidemiological, and clinical evidence demonstrating that the oncogenic potential of HBV may induce the development of HCC both in patients with cirrhosis and in those with a milder liver disease. Chronic HBV infection accounts for approximately 50% of the total cases and for virtually all childhood HCC, and prospective cohort studies showed a 5- to 100-fold increase in the risk of developing HCC among HBsAg carriers compared with uninfected subjects[75]. In spite of this, the role of OBI in the development of HCC in patients with chronic hepatitis due to etiological agents other than HBV, firstly HCV, is still a matter of debate in the scientific community. Using anti-HBc positivity or the presence of HBV DNA in plasma or liver tissue as a sign of OBI, several research groups have investigated the role of OBI in the development of HCC in HBsAg-negative patients with CHC.

The impact of OBI, as detected by the presence of serum anti-HBc, on the development of HCC in patients with CHC

The studies that evaluated the impact of OBI, as detected by the presence of anti-HBc in serum, on the development of HCC are listed in Table 3. In 1996, Chiba *et al*[76] published data from a cohort study on 412 Japanese patients with CHC with or without cirrhosis and showed a higher incidence of HCC in those with OBI than in those without (23.7% *vs* 7.5%, $P = 0.02$). The same authors reported similar

Table 3 The studies evaluating the role of anti-hepatitis B core in the development of hepatocellular carcinoma in surface antigen of hepatitis B virus-negative patients with chronic hepatitis C

First author, year	No. of patients	Country	Type of study	Liver disease	HCC, positive/tested, *n/n* (%)		*P* value
					Anti-HBc$^+$	Anti-HBc$^-$	
Takano 1995[84]	61	Japan	Cohort	CH	9/36 (25.0)	2/25 (8.0)	NS
Chiba 1996[76]	412	Japan	Cohort	CH/cirrhosis	47/198 (23.7)	16/214 (7.5)	0.020
Chiba 1996[77]	204	Japan	Cross-sectional	cirrhosis	92/128 (71.9)	36/76 (47.4)	0.0005
Shiratori 1997[86]	502	Japan	Case-control	CLD	111/263 (42.2)	81/239 (33.9)	NS
IIHCSG 1998[85]	451	Italy	Cohort	CLD	34/206 (16.5)	32/245 (13.1)	NS
Dutta 1999[78]	51	Australia	Case-control	CH/cirrhosis	10/17 (58.8)	7/34 (20.6)	0.010
Marusawa 1999[34]	2366	Japan	Cross-sectional	CH/cirrhosis	363/1047 (34.7)	248/1319 (18.8)	< 0.01
Hiraoka 2003[48]	202	Japan	Case-control	CLD	109/250 (43.6)	93/342 (27.2)	NS
Imazeki 2003[79]	459	Japan	Cohort	CH/cirrhosis	37/160 (23.1)	26/299 (8.7)	< 0.05
Hasegawa 2005[87]	140	Japan	Cohort	CH/cirrhosis	9/64 (14.0)	9/76 (11.8)	NS
Tanaka 2006[80]	74	Japan	Cohort	CLD	13/53 (24.5)	0/21 (0.0)	0.012
Bruno 2007[49]	160	Italy	Cohort	Cirrhosis	29/86 (33.7)	25/74 (33.8)	0.390
Ikeda 2007[81]	846	Japan	Cohort	CH/Cirrhosis	130/392 (33.1)	107/454 (23.6)	IRR: 1.03 (0.66-1.56)[1] IRR: 1.58 (1.12-2.22)[2]
Adachi 2008[82]	123	Japan	Cohort	Cirrhosis	57/96 (59.3)	10/27 (37.0)	0.0039
Alencar 2008[89]	50	Brazil	Cross-sectional	Cirrhosis	5/12 (41.7)	12/38 (31.6)	NS
Miura 2008[94]	141	Japan	Cohort	CH	22/83 (26.5)	11/58 (19.0)	0.700
Ramia 2008[88]	3364	Lebanon	Cross-sectional	CH/cirrhosis/ healthy controls	7/408 (1.7)	2/2956 (0.07)	0.507
Stroffolini 2008[47]	693	Italy	Cohort	Cirrhosis	44/303 (14.5)	57/390 (12.0)	0.900
Ohki 2010[90]	1262	Japan	Cohort	CLD	160/522 (30.6)	179/740 (24.2)	0.630
Lok 2011[43]	273	United States	Case-control	CH/Cirrhosis	38/121 (31.4)	53/152 (35.0)	0.540
Reddy 2013[83]	459	United States	Case-control	CLD	95/229 (41.5)	27/230 (11.7)	0.010
Tsubouchi 2013[91]	400	Japan	Cohort	CLD	24/213 (11.3)	14/187 (7.5)	0.280

[1]Incidence Rate Ratio for HCC in patients with chronic hepatitis; [2]Incidence Rate Ratio for HCC in patients with cirrhosis. CH: Chronic hepatitis; CLD: Chronic liver disease; NS: Not significant; HCC: Hepatocellular carcinoma; HBc: Hepatitis B core.

results in a cross-sectional study on 204 cirrhotic patients[77]. In 1999, a case-control study on 51 Australian patients with CHC with or without cirrhosis showed a correlation between the occurrence of HCC and male gender, lower serum albumin level, and anti-HBc positivity[78]. In the same year, Marusawa *et al*[34] published a study on 2014 patients with CHC with or without cirrhosis and showed that patients with OBI had a significantly higher rate of HCC than those without (34.7% *vs* 18.8%). Similar results were reported in a cohort study[79] on 459 Japanese patients followed up for a mean period of 6.6 years, where the incidence of HCC correlated with the age of the patients, the degree of liver fibrosis, alanine aminotransferase (ALT) levels and anti-HBc positivity. Another Japanese cohort study[80] on 74 CHC patients showed a correlation between the incidence of HCC and anti-HBc positivity. Ikeda *et al*[81] performed a prospective study on 872 Japanese CHC patients and observed in those with liver cirrhosis a significantly higher occurrence of HCC in those with OBI than in those without, a difference not observed in patients with a lower degree of liver fibrosis. Adachi *et al*[82] followed up 123 Japanese cirrhotic patients for a mean period of 53.3 mo and identified as independent predictors of HCC development male gender, higher α-fetoprotein and ALT serum values, and the presence in serum of anti-HBc but not HBV DNA. A case-control study recently conducted by Reddy *et al*[83] in North America on 459 anti-HCV-positive patients with CHC showed a significantly higher frequency of HCC in those with OBI than in those without.

Several studies, however, produced different results. A prospective investigation[84] on 61 CHC patients found no difference in HCC occurrence between groups of patients with or without previous exposure to HBV. The cohort study conducted in 1998 by the Italian IFN-α Hepatocellular Carcinoma Study Group[85] on 451 anti-HCV-positive subjects showed a similar incidence of HCC in anti-HBc-positive and -negative cases. In 1997, a Japanese study on 502 patients found a similar frequency of HCC in anti-HBc-positive and anti-HBc-negative patients[86]. Hiraoka *et al*[48] in 2003 and Hasegawa *et al*[87] in 2005 also published similar data. Likewise, Bruno *et al*[49] demonstrated that anti-HBc positivity was not independently associated with HCC occurrence in 163 Italian consecutive cirrhotic patients with HCV infection followed up for a median period of 10.7 years. Similarly, Stroffolini *et al*[47] found no association between serum anti-HBc positivity and HCC development in a multicenter retrospective cohort study of 693 Italian cirrhotic patients. This association was not found also in two cross-sectional studies, one conducted in Lebanon[88] and one in Brazil[89]. In a cohort study[90] on 1262 Japanese HCV patients, anti-HBc positivity was associated with the development of HCC in a univariate analysis but not in a multivariate analysis considering age and gender as confounding factors. Finally, Tsubouchi *et al*[91] published in 2013 the results of a prospective study on 400 anti-HCV-positive

Table 4 The studies evaluating the role of hepatitis B virus DNA in serum and/or liver tissue in the development of hepatocellular carcinoma in surface antigen of hepatitis B virus-negative patients with chronic hepatitis C

First author, year	No. of patients	Country	Type of study	Liver disease	Sample for HBV-DNA detection	HCC, positive/tested, *n*/*n* (%)		*P* value
						OBI+	OBI-	
Pollicino 2004[39]	226	Italy	Case-control	CH/cirrhosis/ HCC	Liver	45/101 (44.5)	28/125 (22.4)	< 0.001
Tanaka 2004[80]	93	Japan	Cross-sectional	CH/cirrhosis/ HCC	Serum	25/32 (78.1)	25/61 (41.0)	< 0.001
Hasegawa 2005[87]	140	Japan	Cohort	CH/cirrhosis	Serum	2/11 (18.2)	16/129 (12.4)	NS
Squadrito 2006[40]	134	Italy	Cohort	CH/cirrhosis	Liver	8/53 (15.1)	1/81 (1.2)	0.002
Branco 2007[93]	66	Brazil	Cross-sectional	CH/HCC/health controls	Serum/liver[1]	7/10 (70.0)	13/56 (23.2)	0.029
Adachi 2008[82]	123	Japan	Cohort	Cirrhosis	Serum	6/14 (42.9)	60/109 (55.0)	NS
Matsuoka 2008[68]	468	Japan	Cohort	CH/cirrhosis	Serum/liver[1]	29/204 (14.2)	9/264 (3.4)	0.0001
Miura 2008[94]	141	Japan	Cohort	CH	Serum	4/8 (50.0)	29/133 (21.8)	0.0036
Obika 2008[41]	167	Japan	Cohort	CLD	Liver	2/25 (8.0)	10/142 (7.0)	NS
Shetty 2008[42]	44	United States	Cross-sectional	cirrhosis	Liver	12/22 (54.5)	8/22 (36.3)	NS
Lok 2011[43]	83	United States	Case-control	CH/Cirrhosis	Liver	3/16 (18.7)	25/67 (37.3)	NS
Squadrito 2013[69]	94	Italy	Cohort	CH/cirrhosis	Liver	13/37 (35.1)	5/57 (8.1)	< 0.01

[1]OBI assessed with immunochemistry for HBsAg and/or HBcAg. OBI: Occult HBV infection; CH: Chronic hepatitis; CLD: Chronic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

patients and showed no difference in the incidence of HCC and of cumulative liver-related mortality in patients with and without OBI.

The impact of OBI, as detected by the presence of HBV DNA in serum or liver tissue, on the development of HCC in patients with CHC

The studies listed in Table 4 investigated the correlation between HBV-DNA positivity in plasma or in liver tissue and the development of HCC in CHC patients (Table 4). Pollicino *et al*[39] tested for HBV DNA in the tumorous tissue of 73 patients with CHC and HCC and a liver sample of 153 CHC patients used as controls and observed a significant association between OBI and HCC, irrespective of age or gender. In a cross-sectional study published in 2004, Tanaka *et al*[92] demonstrated a significantly higher frequency of cases with HCC in CHC patients with plasma HBV-DNA positivity than in those without. Branco *et al*[93] studied 26 Brazilian CHC patients, 20 with HCV-related HCC and 20 healthy controls, for HBV DNA in serum and for HBsAg and HBcAg immunochemistry in liver tissue and found a higher prevalence of HCC in the 10 patients with OBI than in the 56 without (70% *vs* 23%). Seeking HBV DNA in the liver tissue of 124 CHC patients followed up for a mean period of 82.8 mo, Squadrito *et al*[40] found a significant association between OBI and HCC occurrence, a finding confirmed in a study they published more recently[69]. In 2008, a cohort study[94] enrolling 141 Japanese CHC patients identified OBI as an independent predictor of HCC development.

Some published studies, however, report conflicting data. A prospective study by Obika *et al*[41] on 167 patients with CHC showed a similar incidence rate of HCC over a mean follow-up of 42.5 mo in patients with or without HBV DNA in liver tissue (8% *vs* 7%, respectively). In 2008, Shetty *et al*[42] published a study on 56 patients selected for orthotopic liver transplantation (OLT), 44 of whom underwent OLT. Serum HBV DNA was detected in 28% of the 56, and liver HBV DNA was detected in 50% of the 44. Explant-proven HCC was found in 12 of the 22 (54.5%) patients with OBI and in eight of the 22 (36.3%) without, a difference not statistically significant. Lastly, Lok *et al*[43] tested for HBV DNA in frozen liver samples of 83 CHC patients, 28 with HCC and 55 controls, and found no association between OBI and HCC.

CONCLUSION

The clinical impact of OBI on the natural history of CHC has been extensively investigated, but the available data are conflicting and do not allow for conclusions to be drawn on this topic. One of the main reasons for this inconsistency is the heterogeneity of the methods used to detect OBI. In fact, the detection of HBV DNA in liver tissue of HBsAg-negative subjects can be considered of high sensitivity and high specificity, and that of HBV DNA in plasma of high specificity and moderate sensitivity. In addition, the detection of anti-HBc in serum should be considered of moderate specificity and moderate sensitivity in this setting, although anti-HBc-negative subjects may show HBV DNA in the liver tissue. Furthermore, the variety of diagnostic molecular assays used to identify HBV DNA in plasma and liver tissue of HBsAg-negative subjects possess different sensitivities, bringing considerable heterogeneity in the results. Indeed, in the majority of studies, anti-HBc in serum or HBV DNA in plasma was used to detect OBI, since this method is cheaper, less invasive, and less time-consuming than the detection of HBV DNA in the liver tissue.

Other reasons for the substantial variability in the prevalence of OBI in published studies may be the differences in the extent of the spread of HBV infection in the various geographical areas, the variability in the viral characteristics, and the heterogeneity of the enrolment criteria regarding age, gender,

immunological and ethnic background, and social habits of the subjects examined.

In addition, OBI itself is a virological condition of different origins; most patients having a self-limiting AHB and a minority from the pool of HBsAg chronic carriers, of whom nearly 1% per year clear serum HBsAg. Subjects with OBI of different origins may be present in different proportions in the studies published, and OBI itself may have a different outcome and a different impact on the clinical course of CHC in relation to its origin.

In light of this, we should conclude that the present knowledge on the clinical impact of OBI on the progression of liver fibrosis and on the development of HCC is still insufficient.

In order to reduce the effect of different methods with different sensitivity and specificity used to detect OBI in the published studies, we performed a comprehensive analysis of the studies in which OBI was identified by the detection of HBV DNA in the liver tissue, but the results remained conflicting. In fact, regarding the progression to cirrhosis, we have only three studies, two from the same Italian group[31,69] showing a higher rate of patients with cirrhosis in CHC with OBI than in those without, and one from another Italian group[44] showing no difference. Regarding the development of HCC, six studies were analyzed, three of which were from the same Italian research group[39,40,69], showing a higher rate of patients with HCC in the group of patients with OBI than in those without, whereas the other three studies, one from Japan[41] and two from the United States[42,43], showed no difference. The selection criteria were certainly different from one study to another, but the methods to detect HBV DNA in the liver were similar, albeit not identical. Therefore, the question whether OBI might influence the natural course of CHC remains unanswered.

A strong contribution to defining the clinical impact of OBI could come from a prospective international study considering a large number of HBsAg-negative patients with CHC selected with pre-established criteria and using as sign of OBI the detection of HBV DNA in the liver tissue performed with a highly sensitive technique in a single, high standard laboratory.

No standardized strategy, at least to our best knowledge, is at present recommended for the management of OBI in patients with CHC. In particular, because of the uncertainty surrounding the clinical impact of OBI, it is not clear whether close monitoring is an adequate measure or whether the administration of an anti-HBV nucleot(s)ide to prevent both the progression of fibrosis and the onset of HCC is necessary. In this case, the low cost of the anti-HBV nucleoside lamivudine, which is now obsolete in other HBV treatment settings because of its low genetic barrier and the consequent high risk of inducing viral resistance, might be the drug of choice to suppress the low level of HBV replication characterizing OBI.

In conclusion, some studies indicate that OBI unfavorably affects the progression of liver fibrosis and the development of HCC in patients with CHC, an observation not confirmed in other investigations. The data from prospective studies applying a careful selection of patients and a highly sensitive, standardized method to identify HBV DNA in the liver tissue may help clarify this important issue.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis B virus

Hepatitis B virus burden in developing countries

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Abstract

Hepatitis B virus (HBV) infection has shown an intermediate or high endemicity level in low-income countries over the last five decades. In recent years, however, the incidence of acute hepatitis B and the prevalence of hepatitis B surface antigen chronic carriers have decreased in several countries because of the HBV universal vaccination programs started in the nineties. Some countries, however, are still unable to implement these programs, particularly in their hyperendemic rural areas. The diffusion of HBV infection is still wide in several low-income countries where the prevention, management and treatment of HBV infection are a heavy burden for the governments and healthcare authorities. Of note, the information on the HBV epidemiology is scanty in numerous eastern European and Latin-American countries. The studies on molecular epidemiology performed in some countries provide an important contribution for a more comprehensive knowledge of HBV epidemiology, and phylogenetic studies provide information on the impact of recent and older migratory flows.

Key words: Hepatitis B virus; Molecular epidemiology; Prevention; Developing countries; Chronic hepatitis

Core tip: Hepatitis B virus (HBV) infection is a heavy burden in most developing countries because of its wide spread, particularly in rural areas, and the high cost of

prevention, management, and treatment. Therefore, a greater effort should be made towards implementing universal vaccination programs as they have been demonstrated to be effective in reducing the incidence of acute hepatitis B and the prevalence of hepatitis B surface antigen chronic carriers. In several low-income countries, an improvement in the current knowledge of HBV epidemiology, molecular epidemiology, HBV replication and co-infection with other viruses such as hepatitis C virus and human immunodeficiency virus is strongly desired.

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INTRODUCTION

Nearly 240 million people worldwide carry hepatitis B virus (HBV) infection[1], associated in nearly half of the cases with a chronic liver illness. The progression of liver disease to the more severe forms and the development and complications of hepatocellular carcinoma (HCC) entail a heavy burden for low-income countries. Also political and socio-economic problems make it difficult, at times impossible, to deal with the prevention, management and treatment of HBV infection and associated diseases. This review article focuses on the epidemiology and prevention of HBV infection in low-income countries with an intermediate or high endemicity level.

AFRICA (EPIDEMIOLOGY, MOLECULAR BIOLOGY, PREVENTION)

Epidemiology

Africa is on the whole considered to have a high HBV endemicity. HBV infection is hyperendemic [> 8% of hepatitis B surface antigen (HBsAg) chronic carriers in the general population] only in some sub-Saharan countries such as Nigeria, Namibia, Gabon, Cameroon, Burkina Faso. Other countries like Kenya, Zambia, The Ivory Coast, Liberia, Sierra Leone and Senegal are considered areas of intermediate endemicity (2%-8%), while Egypt, Tunisia, Algeria and Morocco, located in the north of the continent, show a low endemicity level (< 2%)[2]. The prevalences of HBV carriers and genotype distribution in some African countries are listed in Table 1.

The endemicity level varies also in different districts and in different target groups in the same country, *e.g.*, in Burkina Faso, one of the African countries with a high endemicity[3], the HBV overall prevalence is estimated at around 14.5%[4], some authors having reported a level of 12.1% in the health district of Nanoro[5], 18% in blood donors of Nouna, 11% in blood donors and 9.3% in pregnant women in the district of Ouagadougou[6,7]. In Nigeria, HBsAg seropositivity is estimated at around 13.6%, but higher rates have been found in surgeons (25.7%)[8], voluntary blood donors (23.4%)[9] and infants (16.3%)[10]. In Cameroon, recent studies reported an HBV prevalence of 10.1% and 12.1% in blood donors referring to two hospital blood banks[11,12] and of nearly 8% in pregnant women[13-15].

HBsAg-positive age-specific rates were estimated on a global level for 1990 and 2005 using an empirical Bayesian hierarchical model. A 12% prevalence was observed in 1990 in children and adolescents aged up to 19 years in western sub-Saharan African countries, the highest rate documented in the world in this age class, and only slightly decreasing in 2005. In southern sub-Saharan Africa, chronic HBV infection among younger age groups (0-14 years) had increased in 2005, with a prevalence of 8%-9% in females. Also in eastern sub-Saharan African countries, the HBsAg positivity rate had increased in the younger ages over time, whereas no significant changes were detected in the older age groups. An evident decrease in the HBV endemicity was observed in central sub-Saharan Africa, from a high endemicity in the aged 0-34 in 1990 to intermediate values in all ages in 2005. Also in northern Africa and the Middle East regions, the HBV prevalence decreased from 1990 to 2005, particularly among males aged up to 34 years[16].

Of note, the epidemiology in Africa is characterized by a much higher HBsAg prevalence in rural than in urban areas[17,18] and by a greater risk for males of becoming HBV chronic carriers, with a male to female ratio ranging from 1.1:1 to 3:1 and increasing with the increase in age[19-31]. The higher percentage of HBsAg-positive males harboring HBV chronic infection may be the result of differences in tribal and sexual behaviors between males and females[32].

Compared with the adult HBsAg chronic carriers from Southeast Asia, another hyperendemic area, those from Africa show a lower rate of HBeAg positivity. In African countries, 20%-30% of subjects infected by HBV in their early childhood become chronic carriers and only 10% of them remain HBeAg-positive during adolescence. The majority of HBeAg-positive subjects lose HBeAg quickly, at an annual rate of 14%-16%[33]. As is the case in Euro-Mediterranean countries, also in Africa a large majority (> 85%) of patients with a biochemically and histologically active disease are HBeAg-negative[34,35]. In addition, the rate of HBeAg-positive cases found in HBsAg-positive pregnant women was < 1% in Ethiopia, 1.16% in Ghana[36], 1.39% in Nigeria[37], 3.3% in Zimbabwe[38], 4.6% in South Africa[39], 9.5% in Senegal[40], 16.1% in Zambia[41], and 24% in southern Tanzania[42]. The low

Table 1 Prevalence of hepatitis B virus infection and genotype distribution in some African countries

Countries	HBsAg-positive prevalence[4,11]	HBV genotype distribution[66,68-73,75,77]
Burkina Faso	14.5%	A: A1 southern and eastern Africa
Cameroon	10.1%	A2 South Africa
Gabon	9.5%	D: D1 and D7 northern Africa
Ghana	13.8%	E: western and central Africa
Mali	15.5%	Recombinant A/D and A/E
Mauritania	10.9%	
Nigeria	13.6%	
Senegal	13.8%	
Zambia	6.5%	
Zimbabwe	25.0%	

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

rate of HBeAg positivity in HBsAg-positive pregnant women in most African countries correlates with the low rate of perinatal transmission observed in Africa[43].

The data from 18 African countries showed a median HBsAg-positive prevalence of 12.1% in human immunodeficiency virus (HIV)-infected individuals (range 3.9%-70.3%)[44]. In sub-Saharan Africa, the prevalence of patients with HIV/HBV co-infection varies from 0% to 28.4% in different studies[45-49], with a median rate of 3.8% (0%-13%) in pregnant women and 7.4% (1.2%-7.8%) in children and young adults aged from 18 mo to 17 years. Western African countries seem to have the highest co-infection rates (median: 11.5%) in the continent, southern African countries the second highest (median 5.4%), and eastern African countries the lowest (median 4.1%), with a wide variation in single countries[50]. Also the prevalence of cases with occult HBV infection in HIV-infected patients varies largely across the continent, the available information, mostly from southern and western Africa, stating rates from 10% to 33%[51-56].

In African countries, children are at a high risk of acquiring HBV infection. The annual seroconversion rates to HBV markers varied from 10.2%-60.5% in children aged 1-10 years in Somalia, with the highest rate in those with a lower socio-economic condition[23]. The highest rate of children with HBV infection was 15.7% in children aged 5 and 6 years in a study from South Africa[57]. Children acquire HBV infection most frequently by parenteral horizontal transmission[58] from parents or siblings, as clearly demonstrated by phylogenetic analysis in Gambian families where HBV transmission occurred in at least two-thirds of the families investigated[59]. Unsafe sharing in the daily practices of toiletries and sharpening, cutting, scraping or scratching objects accounts for such a high horizontal transmission. In addition, cultural practices like scarification and tattooing and promiscuous sexual activity greatly increase the risk of HBV infection[29,60-63].

HBV transmission through the transfusion of blood or blood products still occurs[58] and is believed to have an epidemiological impact in some areas in sub-Saharan Africa[64]. Over the last decade, the United States President's Emergency Plan for AIDS Relief and the Global Fund have supported blood safety programs in 38 sub-Saharan African countries. The median percentage of HBV markers in blood donations was 7.1% in 2000/2004 and 4.4% in 2010/2011. From 2000/2004 to 2010/2011, 28 (82%) of the 34 reporting countries described a statistically significant decrease in HBsAg marker-reactive donations. Overall, the combined data from the 34 countries showed a 37% decrease in the HBsAg-reactive donations[65].

Molecular epidemiology

Five HBV genotypes are more frequently detected In Africa, A, B, C, D and E (Table 1)[2]. Despite the limited number of studies, a trend in their distribution is emerging. Genotype A predominates in southern and eastern Africa, genotype D in northern Africa[2] and genotype E in the vast region from Senegal to Namibia and eastward to the Central African Republic. HBV/E is the most frequent genotype found in the Central African Republic, the Democratic Republic of the Congo, Benin, Togo and Nigeria[2,66,67]. Recombinants of HBV genotypes have also been detected, an A and E recombinant in Cameroon[66] and western Africa[67] and an A and D recombinant in healthy black African adults positive for hepatitis B surface antibody alone[68].

Most of genotype A sequenced in Africa belongs to subgenotype A1, which is mainly found in southern and eastern Africa, including South Africa, Malawi, Tanzania, Uganda, the Democratic Republic of the Congo and Somalia. This subgenotype has been frequently detected also in southern Asia (India, The Philippines, Bangladesh, Nepal), supporting the hypothesis that this subgenotype was introduced to Asia through the intensive trade and frequent travels from eastern Africa[69-73].

Subgenotype A2, mainly isolated in South Africa, resembles some European isolates and the hypothesis that Portuguese sailors probably introduced this subgenotype to Europe in the 16th and 17th century has been formulated[73]. Genotype E prevails in native populations of western and central Africa[2]. All genotype E strains have the same characteristic, an in-frame deletion of three nucleotides in the 5' pre-S1 region, a signature pattern of amino acids in the pre-S1 region and a serological subtype formulated as *ayw4*. The low genetic diversity over large geographical areas suggests that HBV/E may have a short evolutionary history and a recent introduction to African countries[2,74].

HBV genotype D is the most prevalent in northern Africa, particularly subgenotypes D1[75,76] and D7[76,77], but it is also diffuse worldwide. A recent comprehensive reconstruction of the phylogeography of HBV genotype D in the European Mediterranean basin indicates that it originated in the second half of the 19th century in

India[78,79].

Prevention

Vaccination is essential to control HBV infection. Thanks to the Expanded Programme on Immunization started in 1995 in some African countries, such as South Africa, the monovalent anti-HBV vaccine continues to be administered at 6, 10, and 14 wk of age and the rate of HBV infection and HCC in children shows a clear tendency to decrease[80].

HBV vaccination in HIV-positive African populations provides a moderately lower response rate than in the general African populations, but, as in other countries, revaccination of non-responders increases the response rate to 95%[81].

ASIA (EPIDEMIOLOGY, MOLECULAR BIOLOGY, PREVENTION)

Epidemiology

Southwestern Asia, also known as the Arabian region, accounts for 10% of the Asian territory. The Arabian peninsula, including Saudi Arabia, Yemen, Oman, Bahrain, the United Arab Emirates (UAE) and Kuwait[82-84], shows an HBsAg-positive prevalence ranging from 1.5% to over 8%[16]. In particular, this prevalence ranges from 1.5% to 2.6% in Saudi Arabia, is reported to be 5.1% in blood donors in Yemen, the poorest country of the Arabian peninsula, 3.5% in volunteer blood donors in Kuwait and to range between 2% and 7% in the general population in UAE[85-88]. The Levant (Sham) Arabian region comprises Syria, Iraq, Lebanon, Jordan and the Gaza Strip. The HBsAg-positive prevalence is 0.6% in the general population in Iraq[89], 1.6% in volunteer blood donors in Lebanon[90] and 1.4 % in blood donors in Jordan[91]. No data are available for Syria at present, apart from its classification as a geographical area with an intermediate endemicity in the report by Lavanchy[86]. The HBsAg-positive prevalence in the Gaza Strip is 3.5% in the general population and 3.8% in blood donors[92]. Arab countries have implemented the WHO-recommended Expanded Programme on Immunization, and HBV vaccination programs started in these countries have now covered a large proportion of their population, successfully reducing the HBV endemicity. In Saudi Arabia, the first Arab country to adopt an HBV vaccination program[93], a steady decline in the HBsAg-positive prevalence has been observed in children aged 1-12 years, from 7% in 1989, to 0.31% in 1997 and 0% in 2008[94,95].

In Cambodia, one of the western Pacific countries, the HBV prevalence was 4.6% in the adult population[96] and 6.3% in blood donors (Ministry of Health in Cambodia, 2013, unpublished. data). In this country, high anti-HBc rates have been reported, 58.6% and 72.4% in different studies[97,98], suggesting a principal role played in the past by horizontal transmission in childhood and adulthood.

The HBsAg-positive prevalence was 3.6% in subjects aged 18-79 years in Singapore in 2010, and HBeAg was detected in 4.2% of the HBsAg-positive cases. The national childhood HBV vaccination program adopted in this country has shown a great impact in reducing the spread of HBV infection[99].

In China, thanks to the universal HBV immunization program of newborn babies initiated in 1992, the prevalence of HBsAg carriers decreased from 9.8% observed in 1992 to 7.18% registered in 2006[100]. Of note, the vaccination coverage rate at the end of 2005 was 20% lower in rural areas than in the urban areas, a difference that has steadily decreased in recent years. Despite the suboptimal coverage, the prevalence of anti-HBs was higher in fully immunized children (63.2%-74.3%) than in non-immunized subjects (21.1%-34.8%)[101]. As a result of the universal HBV vaccination campaign, China has gone from a high to an intermediate endemicity level in a short period of time[102]. At present, however, the HBV prevalence in some high-risk groups is very high, *e.g.*, 11.9% in hemodialysis patients[103] and 12.5% in HIV-positive subjects[104].

In South Korea the HBsAg-positive seroprevalence is 4%, slightly higher in the southern than in the central provinces. In the last decade, however, the universal vaccination program has brought about an impressive reduction in HBsAg positivity documented in the younger population, from 2.2% to 0.12%[105,106].

In Kazakhstan, an HBV seroprevalence of 3.8% has been documented, with a peak in the adult population aged 30-49 (6.3%) and lower rates in the aged 10-29 (2.5%) and in subjects over 50 (1.7%)[107].

In India, the estimated HBsAg-positive prevalence is 3.1% in the non-tribal population and 11.85% in tribal populations[108], with wide geographical variations within this subcontinent due to differences in socioeconomic status, religion, culture and tribal practices.

The prevalences of HBV infection and genotype distribution in some Asian countries are shown in Table 2.

Molecular epidemiology

In the Arabian countries HBV genotype D predominates, particularly, subgenotypes D1 and D3[109]. Patients living on the northern coast of the Persian Gulf are infected mainly with HBV subgenotype D1, spread widely by ancient migrations from Iran, Syria, and Turkey[110].

In China, HBV genotypes B and C, and in particular subgenotypes B2 and C2, predominate, with some geographical differences. Genotype B is more frequent in southern China and genotype C in the north of the country. In some regions of northern China subgenotype C2 is predominant, whereas subgenotype C1 is more frequent than C2 in southern China. Recombinant C/D1 and C/D2 have been found

Table 2 Prevalence of hepatitis B virus infection and genotype distribution in some Asian countries

Countries	HBsAg-positive prevalence[16,85-92,96,99,105-108]	HBV genotype distribution[109-118,121,123,125]
Cambodia	4.6%	A: India A1 India B: China
China	7.18%	B2 southern China
Gaza Strip	3.5%	C: China
India	3.7%	C1 southern China, India
Iraq	0.6%	C2 northern China
Jordan	1.4%	D: Arabian countries and India
Kazakhstan	3.8%	D1 Persian Gulf (Iran, Syria,
Kuwait	3.5%	Turkey), India, Pakistan
Saudi Arabia	1.5%-2.6%	D2, D3, D4, D9 India
Singapore	3.6%	C/D1-CD2 western China
South Korea	4.0%	
United Arab Emirates	2%-7%	
Yemen	5.1%	

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

Table 3 Prevalence of hepatitis B virus infection and genotype distribution in some eastern European countries

Countries	HBsAg prevalence[133-138,142-145,147,148]	HBV genotype distribution[150-156,160,161]
Albania	> 9%	A: Eastern Europe (Poland, Czech Republic, Bulgaria, Hungary[1]) A2 Bulgaria D: Southeastern Europe (Russia, the Baltic region, Belarus, Romania, Hungary, Serbia, Croatia, Lithuania, Romania, Bulgaria)[1] D1 Bulgaria D2 Albania, Russia, Estonia, the Siberian and eastern part of the former USSR
Bulgaria	3.80%	D3 Serbia
Croatia	1.7%-15.8%	[D: prevalent genotype
Poland	3.91/100000[1]	(70%-80%)]
Romania	5.59%	1A,D: equal distribution
Russia	7.6/100000	
Serbia	4.4%-13%	

[1]Incidence/year. HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

to be predominant in the Qinghai-Tibet Plateau and in western China, indicating that the spread of these two recombinants may have an ethnic origin[111-118]. Other HBV subgenotypes, such as C5 and C7, possibly introduced from the southeastern Asian countries, have been infrequently detected in China[119]. Compared with HBV subgenotype B, subgenotype C shows a lower replicative activity in young patients and harbours higher frequencies of HCC-associated mutations[120].

In Pakistan HBV genotype D predominates, particularly subgenotypes D1 and D3 and a B/D recombinant plays a marginal role, being responsible for 3.5% of the cases. It has been suggested that genotype D achieved its wide distribution in ancient times associated with the ancient history of the civilizations in this region[121].

Taking into consideration the HBV-host co-evolution, the diverse Indian population provides an excellent opportunity for further studies to investigate some underpinnings of the HBV diversity. Of the three HBV genotypes found in India, namely D, A and C, genotype D is predominant, whereas all the HBV/A and HBV/C isolates discovered in India belong to subgenotype A1 and C1, respectively; the genotype D strains are divergent and classified into 5 distinct subgenotypes, D1, D2, D3, D5 and D9, with a different geographical distribution[122-125].

Prevention

The WHO estimates that Asia is the continent with the highest rate of HBsAg carriers in the world, with an overall prevalence in the adult population of over 8%. In order to prevent HBV infection and its associated diseases, several Asian countries have started vaccination programs[126]. An extensive program (China GAVI Hepatitis B Immunization Project) was started in China in 2002 in all the Chinese provinces to prevent HBV transmission to newborn babies in order to decrease over time the circulation of HBV in the country and reduce the heavy burden of liver cirrhosis and HCC. Although the program has not yet reached some rural areas, HBV vaccination covers more than 75% of newborn babies and the rate of HBsAg positivity has decreased from 10% to 1%[127]. This project has certainly required a strong political, social and economic commitment, but the results obtained to date are truly impressive.

EASTERN EUROPE (EPIDEMIOLOGY, MOLECULAR BIOLOGY, PREVENTION)

Epidemiology

The low number of epidemiological studies on HBV infection performed in eastern Europe does not allow conclusive statements to be made on the spread of HBV infection and on the level of application of universal vaccination in this large geographical area. The available data from eastern European countries show higher HBsAg-positive prevalences than in western Europe[128-131] (Table 3), but the ongoing universal HBV vaccination campaign in rural and urban areas of the single countries will reduce this gap in the near future. In fact, in a recent study from Bulgaria, the HBsAg-positive prevalence in individuals aged 19 or less, targeted by HBV vaccination, was significantly lower than that found in non-vaccinated individuals aged over 20 (1% *vs* 4.8%)[132]. The HBsAg-positive seroprevalence in the general population was 3.8% in studies performed in Bulgaria[133,134], 5.6% in

Table 4 Prevalence of hepatitis B virus infection and genotype distribution in some Latin American countries

Countries	HBsAg prevalence[166-169]	HBV genotype distribution[177,178]
Mexico, Honduras, Nicaragua, Costa Rica, Panama, Uruguay, Chile, Argentina, Peru, northern Colombia	Low prevalence (< 2%)	F: F1 Central America eastern South-America F2 Venezuela, Brazil F3 Central (Panama) and northern (Colombia and Venezuela) Latin America F4 Bolivia and Argentina (F genotype: prevalent) H: Amerindians and Mestizios in Mexico A, D: Gualalajara, Jalisco, Mexico, Argentina
Central America: Guatemala, El Salvador, Honduras, Haiti, Dominican Republic, Puerto Rico South America: Ecuador, French Guyana, Suriname, south Brazil	Intermediate prevalence (> 2%, < 8%)	
Northern Brazil, southern Colombia, Peru, northern Bolivia	High prevalence (> 8%)	B, C: dispersed among Latin America populations (due to Asian immigrants)

HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

Romania[135] and from 4.4%-13% in different studies in Serbia[136-138], with wide variations within single countries that reflect the different socio-economic conditions between rural and urban areas[135]. In these studies, males showed higher rates of HBsAg positivity than females.

Although introduced in 1995, HBV vaccination has not as yet significantly reduced the high HBV endemicity level in Albania[139-141], where HBsAg positivity in the general population is over 9% and the overall risk of becoming infected exceeds 60%[142-145]. In Poland, an incidence of acute hepatitis B of almost 4 cases per 100000 inhabitants was registered both in 2011 and 2012, suggesting a stable spread of HBV infection in this country[146]. In the adult population in Croatia, HBV seropositivity increased with the increase in age, from 1.7% to 15.8%, and was higher in subjects from rural areas than from urban areas (10.7% *vs* 6.1%)[147]. The incidence of acute hepatitis B in Russia was 7.6 per 100000 inhabitants in 2009, with wide variations across the country[148].

Molecular epidemiology

HBV genotypes A and D are those most frequently detected in eastern Europe (Table 3)[149], genotype D being responsible for 70%-80% of the HBV infections occurring in the northern and central areas and in eastern Mediterranean countries[128,150-153]. In fact, HBV genotype D predominates in Romania (67%), Lithuania (54%), Serbia (82%), Croatia (80%), Albania (92%) and Russia (93%), whereas genotype A predominates in Poland (77%) and in the Czech Republic (67%), two countries with similar ethnic backgrounds and a small proportion of immigrants (3%-4%)[79,128,152,154,155].

HBV genotype A and D, and subgenotypes D1 and A2 in particular, are those more frequently detected in Bulgaria[156]. Using a phylodynamic approach, the beginning of the spread of D1 in this country dated back to the early 1980s[78,156], whereas the strains analyzed of subgenotype A2 dated back to 1996. HBV genotype A is frequent in central and northern Europe, where the HBV spread is mainly sustained by sexual transmission[157-159], and in Bulgaria, which has a crossroads position between western and eastern Europe favoring the introduction of new subgenotypes[156].

More than 70% of Albanian HBsAg carriers are infected with HBV D2 subgenotype, suggesting an epidemiological relationship between Albania and northeastern European countries of the former USSR, rather than from other Mediterranean countries, where HBV subgenotypes D1 and D3 predominate[79,160,161].

Hungary shows an almost equal distribution of HBV genotypes A and D, probably due to its central position between western and eastern Europe[152].

Prevention

In 2009, Nardone *et al*[162] published a report on the HBV epidemiology in 10 European countries in relation to the application of the vaccination policies. At the time of publication of this report, HBV universal vaccination programs recommended by the WHO were in progress in different countries of eastern Europe (the Czech Republic, Romania and Slovakia), but coverage differed between countries, most probably reflecting the difficulty to reach people living in rural areas in some countries (Romania, Slovakia).

In Poland, HBV vaccination of newborn babies is active and no new HBV cases in childhood and adolescence have been registered, whereas non-vaccinated subjects aged 45-49 years still show a high rate of acute HBV infection[163].

LATIN AMERICA (EPIDEMIOLOGY, MOLECULAR BIOLOGY, PREVENTION)

Epidemiology

The information on the HBV epidemiology in Latin American countries is scanty and fragmentary, but it has been estimated that 7-12 million Latin Americans carry HBV chronic infection[164,165]. The rate of HBsAg-positive subjects varies between countries (Table 4)[166], the highest values being detected in the 20-40 age class as a possible consequence of a major role played by horizontal transmission[163].

More recently, some tropical Latin American areas such as Panama, Colombia and Venezuela shifted from an intermediate to a low endemicity level[166-169]. In

addition, countries with a low HBV endemicity show a high rate of anti-HBc positivity in HBsAg-negative subjects, a clue to the more extensive exposure to HBV in the past[170,171]. A slight decline in the HBsAg-positive prevalence was observed from 1990 to 2005 in Andean Latin American countries, whereas a slight increase was reported in southern Latin America in the same period[16].

Molecular epidemiology

HBV genotypes F and H predominate in indigenous populations of Latin America, whereas genotypes A and D have been introduced from European and African populations[172,173]. Four subgenotypes of HBV genotype F (F1-F4) have been identified, predominating in Central America and frequent in Amerindians in all countries of South America[174-176] (Table 4), and HBV genotype H in Amerindians and in Mestizos in Mexico[177,178]. Genotypes F and H show a close phylogenetic relationship, suggesting an introduction of F/H ancestral strains before European colonization[174].

Introduced by European colonization, HBV genotypes D and A have been detected in nearly 35% and 5% of HBsAg-positive subjects of the urban population of Guadalajara and Jalisco (Mexico), respectively, while in various cities of Argentina they have been documented with frequencies ranging from 22% and 45%, respectively[177-179].

Genotypes B and C, introduced by Asian immigrants, are sporadically detected[180,181].

Worthy of note is that liver cirrhosis and hepatocellular carcinoma are rare in Mexicans, indicating that the immune response and course of liver disease in the Mexican native population may differ from that described in other geographical areas worldwide[182,183].

Prevention

Unfortunately, the universal vaccination programs remain unaffordable for most South American countries[184,185]. Where applied, however, they have achieved important epidemiological results, *e.g.*, in the Colombian Amazon, where the rate of HBsAg positivity dropped from 9% to 2% in children after eight years of application[186].

FINAL COMMENTS

Although of reduced impact in several countries due to the HBV universal vaccination programs started in the nineties', HBV infection still entails a heavy socio-economic burden in several developing countries.

Vaccination programs should be extended without delay to cover rural areas of the countries where HBV vaccination is showing its efficacy in reducing the spread of HBV infection. Countries still unable to adopt a universal immunization program for newborn babies should receive support from international health organizations to implement this.

At present, the high cost of effective nucleo(t)side analogues, namely entecavir and tenofovir, to treat HBV infection and its correlated diseases is a strong handicap for most developing countries where numerous patients await treatment.

In addition, the information on the HBV epidemiology is scanty in several low-income countries and needs to be extended to cover information on HBV replication, co-infection with HCV and HIV, molecular epidemiology, phylogenies and clinical aspects.

In Africa, co-infection with HIV is a further problem that requires a therapeutic approach with the most appropriate combination therapies.

In Asia, a high viral load and a high prevalence of HBeAg-positive patients characterize HBV infection, and make the achievement of viral suppression more complex.

In Eastern Europe and South America, more epidemiological, virological and phylogenetic information is needed and further implementation of the vaccination programs to cover all the rural territories.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis B virus

Rapid and quantitative detection of hepatitis B virus

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Abstract

Despite availability of a universal vaccine, hepatitis B virus (HBV) infection has a huge impact on public health worldwide. Accurate and timely diagnosis of HBV infection is needed. Rapid developments have been made in the diagnostic and monitoring methods for HBV infection, including serological and molecular assays. In clinical practice, qualitative hepatitis B surface antigen (HBsAg) testing has long served as a diagnostic marker for individuals infected with HBV. More recently, HBsAg level has been used to predict treatment outcome when determined early during treatment or at baseline. However, identification of HBV DNA positive cases that do not have detectable HBsAg has encouraged the application of molecular tests. Hence, combination of quantitative detection of HBV DNA and HBsAg can be used to discriminate patients during the course of HBV infection and to monitor therapy. This article reviews the most commonly used quantitative methods for HBsAg and HBV DNA.

Key words: Hepatitis B virus; Biosensor; Polymerase chain reaction; Isothermal amplification methods; Quantitative assay

Core tip: The combination of quantitative detection of hepatitis B surface antigen (HBsAg) and hepatitis B virus (HBV) DNA can be used to classify individuals during the course of HBV infection and to monitor therapy. The most popular platforms for HBsAg detection are based on chemiluminescent microparticle immunoassay, while polymerase chain reaction based methods are widely used for HBV DNA assay. Recently, isothermal amplification and biosensors offered a lower cost and more rapid alternative for HBV quantification. This article reviews the most commonly used quantitative methods for HBV.

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INTRODUCTION

Hepatitis B virus (HBV) is an enveloped virus with a small (3.2 kb) partially double-stranded DNA genome that causes acute and chronic infections[1]. The impact of HBV infection on public health is enormous, with an estimated prevalence of 2 billion infected and 360 million chronically infected[2]. The diagnosis of HBV infection relies heavily on serological and molecular tests. Serological tests use serum-based blood tests that can be analyzed by enzyme immunoassays either qualitatively or quantitatively[3]. The tests identify virus-encoded antigens and their corresponding antibodies: hepatitis B surface antigen (HBsAg), anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe, and antibodies to hepatitis core antigen (anti-HBc). In contrast, the molecular tests focus on quantitative viral load, genotyping, drug resistance mutations, and core promotion/pre-core mutation assays.

In clinical practice, qualitative assays for HBsAg have served as diagnostic markers for patients with HBV infection. More recently, quantitative methods for HBsAg have been used to predict treatment outcome when determined early during treatment or at baseline[4]. However, identification of HBV-DNA-positive cases, which do not have detectable HBsAg, is greatly encouraging for the application of molecular testing. This article reviews the most commonly used quantitative detection methods for HBsAg and HBV DNA (Figure 1).

QUANTITATIVE METHODS FOR HBsAg

Since the discovery of HBsAg in 1965[5], it has served as a biomarker for the diagnosis of HBV infection. Methods for HBsAg detection were first described in the 1970s using electron microscopy, radioimmunoassay, and enzyme immunoassays[6-8], which were cumbersome, labor intensive, and restricted to a research setting. Since then, various diagnostic methods have been developed for quantitative HBV detection.

In the early 1990s, HBsAg quantification was considered to be a simple, promising, and inexpensive method to monitor viral replication in chronic hepatitis B (CHB) patients who were receiving interferon (IFN) therapy[9]. HBsAg quantification is associated with the concentration of covalently closed circular DNA (cccDNA), the persistent intrahepatic form of HBV DNA[10,11]. The amount of circulating HBsAg is hypothesized to be predictive for response to antiviral therapy. Currently, standard quantitative HBsAg assays have been developed, which are fully automated and have high throughput. Two commercially available assays are briefly introduced here.

The Architect HBsAg QT (Abbott Laboratories, Abbott Park, IL, United States) is an automated chemiluminescent microparticle immunoassay method, which is the most widely used assay in clinical practice[12]. The Architect HBsAg QT assay is a two-step immunoassay with flexible assay protocols, referred to as Chemiflex, for quantitatively measuring HBsAg concentrations in serum and plasma[13]. The Architect HBsAg system can detect as low as 0.2 ng/mL HBsAg with a dynamic range of 0.05-250.0 IU/mL (1 IU/mL is equivalent to 1-10 ng/mL HBsAg)[14]. The assay is capable of processing up to 800 tests per hour.

Elecsys HBsAg Ⅱ (Roche Diagnostics, Indianapolis, IN, United States) is another popular assay for HBsAg quantification[15]. Elecsys HBsAg Ⅱ is a "sandwich" assay with a total testing time of 18 min. The results are reported as a cutoff index (signal sample/cutoff). The sample is considered nonreactive when the index is < 0.9, while samples with index value > 1.0 are interpreted as reactive[15].

These two methods can be confidently used for HBsAg quantification for the most prevalent HBV genotypes. These tests are easy to use, inexpensive, and have a rapid turnaround time; the analytical performance of the assays is generally satisfactory.

QUANTITATIVE METHODS FOR HBV DNA

With the increased prevalence of serologically negative HBV infections (HBeAg negative CHB and occult HBV infection) and the rapid advent of diagnostic escape mutants, the detection of HBV DNA has gained more attention in clinical medicine[16]. The detection of HBV DNA in peripheral blood is a reliable marker of HBV activity, while high levels of HBV DNA are associated with a higher incidence of hepatocellular carcinoma (HCC) and more rapid progression to cirrhosis[17]. In addition, HBV DNA detection is beneficial in routine clinical practice to identify individuals who need anti-viral treatment and provide them the most suitable therapy[18]. Nowadays, a variety of molecular technologies have been used in HBV DNA quantification, such as ultraviolet (UV) spectrophotometry, real-time polymerase chain reaction (PCR), digital PCR, isothermal amplification methods, and biosensors[19-22]. Here, we review the rapid and quantitative methods that have been used for HBV DNA detection.

UV spectrophotometry

The aromatic rings of the bases absorb UV light with a maximum peak at 260 nm. When a beam of UV light shines through a sample containing DNA or RNA, the amount of UV absorption by the sample depends on the DNA or RNA concentration. The amount of UV light absorbed by a series of standard DNA amounts is measured to calibrate the technique, and the ratio of UV light absorbed by the unknown sample is measured and plotted on the calibration curve to deduce the concentration of DNA or RNA.

NanoDrop instruments (Thermo Scientific, Wilmington, DE, United States) are based on UV

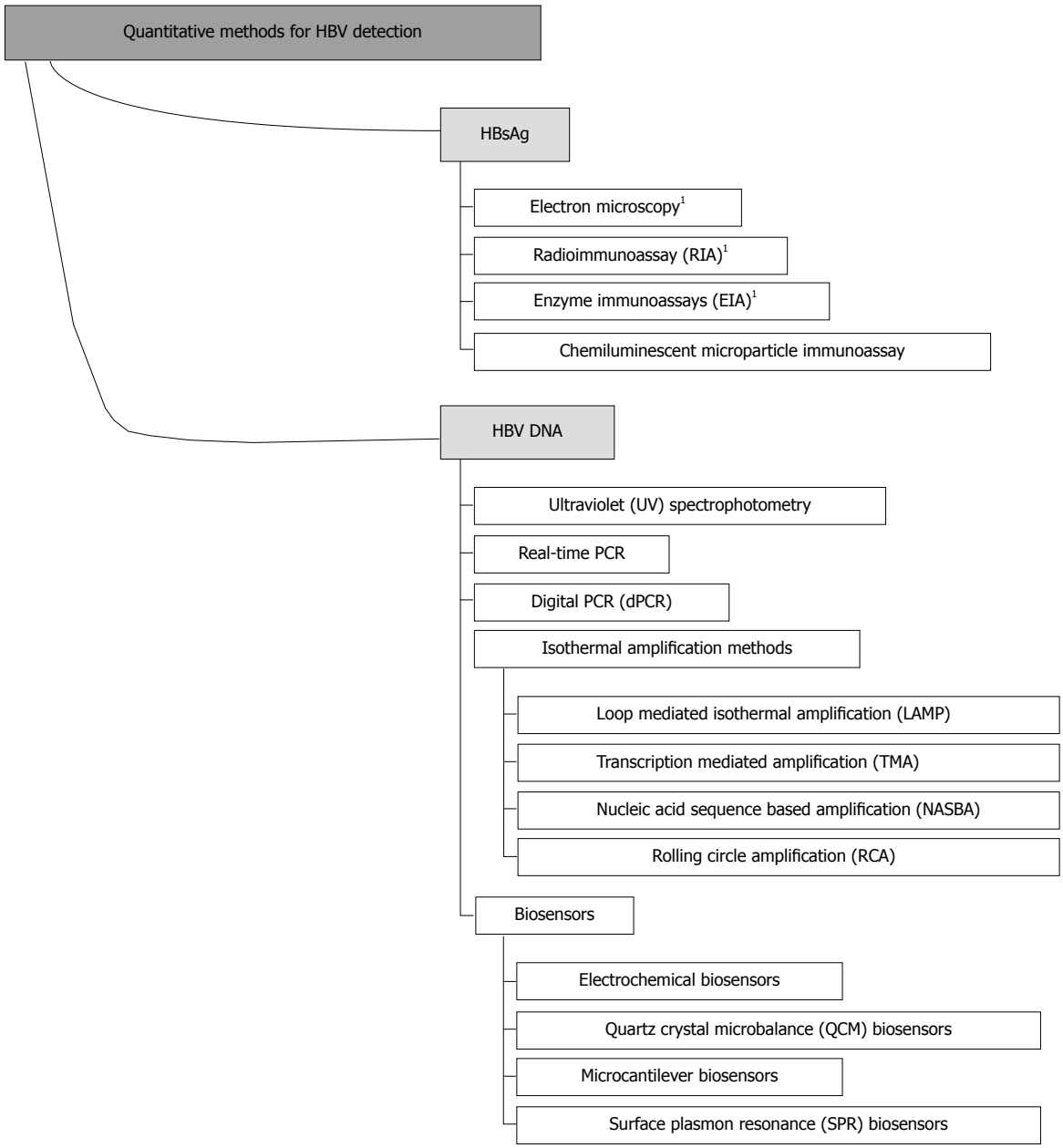

Figure 1 Quantitative methods for hepatitis B virus detection. [1]These methods are qualitative or semi-quantitative for HBsAg. HBsAg: Hepatitis B surface antigen.

spectrophotometry and utilize a patented sample-retention system that allows the quantification of DNA or RNA from 1-2 μL samples[23]. The usable concentration ranges are 0.4-15000 ng/μL. No specific sample preparations are needed for NanoDrop instruments[23].

Real-time PCR

Since developed in 1983 by Kary Mullis[24], PCR has become an essential tool for molecular biologists, and its application in nucleic acids detection system has revolutionized the quantitative analysis of DNA and RNA. PCR techniques have rapidly evolved over the last few years, and their quantitative applications have favored the development of real-time PCR. Real-time PCR follows the general principle of conventional PCR, and its key feature is that the amplified DNA is detected as the reaction progresses in real time. This new approach has a broader dynamic range compared to conventional PCR. The most commonly used reagents for real-time PCR are TaqMan probes[25]. TaqMan probes are hydrolysis probes that were designed in 1991 to increase the specificity of quantitative PCR[26]. Figure 2 outlines the reaction mechanism of real-time PCR based on TaqMan probes.

In the study by Abe *et al*[27], a sensitive, accurate, and reproducible assay for HBV DNA quantification based on real-time PCR using TaqMan probes was reported. Their results demonstrated that the limit of detection was as few as 10 copies/reaction, with a

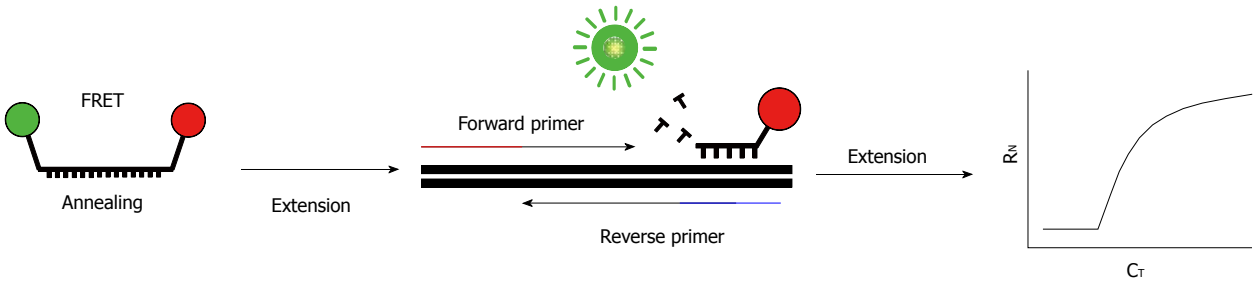

Figure 2 Reaction mechanism of real-time polymerase chain reaction based on TaqMan probe technology. TaqMan probe is an oligo-nucleotide probe that has a fluorescent reporter at the 5' end and a quencher attached to the 3' end. Once hybridized to the target sequence during annealing, TaqMan probe is cleaved by DNA polymerase, which separates the fluorescent reporter from the quencher. Once they are separated, the signal is emitted and detected in the real-time machine. The intensity of fluorescence is proportional to the amount of PCR product produced. FRET: Fluorescent resonance energy transfer.

linear standard curve between 10 and 10^8 DNA copies/reaction. The coefficient of variation for both inter- and intra-experimental variability indicated remarkable reproducibility.

Sitnik *et al*[28] developed a real-time PCR assay for HBV DNA quantification with TaqMan and minor groove binder (MGB) probes. In this assay, primers and probes were designed using an alignment of sequences from all HBV genotypes in order to amplify all the genotypes equally. The assay had a dynamic range from 50 to 10^8 IU/mL.

Although many laboratories have developed in-house real-time PCR-based assays, all of them had a lack of quality control and standardization[3], which restricted their application in clinical diagnosis. The major diagnostics companies also provide commercial quantitative assays. These assays vary in their detection limit and dynamic range: the first version suffered from a narrow dynamic range from 10^2 to 10^5 copies/mL, while the detection limit of the newer assay is lower than 50 IU/mL with a dynamic range of approximately 10^8 IU/mL[29-32]. Recently, the World Health Organization has established a universal standard for HBV DNA quantification measured in IU/mL, with the purpose of correlating different results using a single reference unit[33]. However, significant variability in quantification among different assays can occur randomly in spite of the standardization of reporting units and the generally good correlation between different assays[34,35]. Hence, patients are suggested to be monitored by a single assay[1].

Digital PCR

Digital PCR, developed by Vogelstein *et al*[36], is a refinement of conventional PCR methods that can be used to quantify and clonally amplify nucleic acids directly, including DNA, cDNA, and RNA. The key difference between digital and conventional PCR lies in the measuring method; digital PCR is a more precise method than conventional PCR[37]. A sample is partitioned in digital PCR so that individual nucleic acid molecules within the sample are localized and concentrated within many separate regions. The partitioning of the sample allows for estimation of the number of molecules, by assuming that the molecular population follows a Poisson distribution. Hindson *et al*[38] developed a fundamentally distinct method of partitioning, droplet digital PCR, which partitions a sample into 20000 droplets and provides digital counting of nucleic acids.

High-sensitivity techniques distinguish differences in the number of HBV copies among samples, especially in cases of low copy number (*e.g.*, nucleic acids extracted from tissues). Huang *et al*[39] applied droplet digital PCR to measure the number of HBV copies in formalin-fixed paraffin-embedded (FFPE) HCC tissue. A total of 131 HCC FFPE samples with different tumor stages and clinical features were classified by their serological tests. The number of HBV copies were successfully determined by droplet digital PCR for all FFPE tissues, with copy numbers ranging from 1.1 to 175.5 copies/μL. These results showed that droplet digital PCR improved the analytical sensitivity and specificity of nucleic acids measurement to a single-molecule level and was suitable for HBV DNA quantification.

Isothermal amplification methods

PCR-based assays are the most widely used methods for HBV DNA quantification; however, they need a thermo-cycling machine to separate DNA strands and amplify the fragments[21]. Isothermal amplification methods are carried out at a constant temperature, and do not require a thermal cycler. The isothermal amplification methods have been developed according to new findings in the molecular biology of DNA/RNA synthesis and *in vitro* nucleic acid amplification function of some accessory proteins. Here, we describe several isothermal amplification methods that have been used to quantify HBV DNA.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a single-tube technique for DNA amplification[40]. LAMP may be combined with a reverse transcription step to allow the detection of RNA[41]. In LAMP, the target sequence is amplified at a constant temperature of 60 ℃-65 ℃ using either two or three sets of primers and a polymerase with high activity of strand

displacement and replication activity. An additional pair of loop primers can accelerate the amplification[42]. Due to the specific nature of the primers, the amount of DNA products in LAMP is considerably higher than in PCR-based amplification[42]. Compared with PCR, LAMP was less sensitive to inhibitors in complex samples, such as blood, due to the use of a different DNA polymerase. However, complex primer design has been considered as a weakness of LAMP, and it may limit its application in some aspects of molecular biology[21,41].

The original article on LAMP, which was published in 2000 by Notomi *et al*[40], showed that 600 and 6000 copies of HBV DNA were detected at 13 and 11 min, respectively, which indicated a high specificity and efficiency for HBV detection. Cai *et al*[43] developed an accurate and rapid real-time fluorogenic LAMP protocol to quantify HBV. Their study demonstrated a dynamic range of eight orders of magnitude, a lower detection limit of 210 copies/mL, low inter-assay and intra-assay variability (4.24%-12.11%), and excellent correlation with real-time PCR (R^2 = 0.96). Similar LAMP assays have been reported by others, indicating that LAMP may be useful in the future as a low-cost alternative for HBV DNA quantification[44,45].

Transcription-mediated amplification

Transcription-mediated amplification (TMA) is an isothermal amplification technique used in molecular biology research and in clinical laboratories for the rapid diagnosis of infections. In contrast to PCR, this method involves RNA transcription (*via* RNA polymerase) and DNA synthesis (*via* reverse transcriptase). There are several more differences between TMA and PCR: (1) TMA is isothermal; (2) TMA produces RNA rather than DNA amplicons. Since RNA is more labile in a laboratory environment, this reduces the possibility of carry-over contamination; and (3) TMA produces 100-1000 copies per cycle (PCR only produces two copies per cycle), which results in a 10 billion-fold increase in nucleic acid products within 15-30 min[46].

Kamisango *et al*[47] developed a sensitive and quantitative assay using TMA and hybridization protection assay for the detection of HBV DNA in serum. The assay achieved a detection range of 5 × 10^3 to 5 × 10^8 genome equivalents/mL. It takes about 5 h to complete a moderately sized manual assay. Ide *et al*[48] noted that TMA was more useful in understanding the changes of HBV DNA level than branched DNA signal amplification assay in lamivudine-treated CHB patients. Kubo *et al*[49] examined the usefulness of TMA for evaluation of the active degree of hepatitis and estimation of recurrence after resection of HBV-related HCC.

Nucleic acid sequence-based amplification

Nucleic acid sequence-based amplification (NASBA) is similar to TMA, which was developed by Compton[50] in 1991 to amplify RNA sequences. This technique can also be used for amplification of DNA with modifications in the basic method, such as primer design, sample extraction, and template denaturation[16]. Compared with PCR, major advantages of NASBA are: (1) it works under isothermal conditions - usually at a constant temperature of 41 ℃; and (2) it is more rapid and sensitive than PCR in medical diagnostics[51].

Yates *et al*[52] developed an HBV DNA quantification system based on amplification with NASBA and real-time detection with molecular beacon technology. The detection range of the assay is 10^3-10^9 copies/mL in plasma or serum, with good reproducibility and precision. Deiman *et al*[53] used NASBA, including a restriction enzyme digestion for HBV DNA amplification, and found that the sensitivity of normal NASBA was improved 100-1000 times when restriction enzyme digestion was performed prior to amplification. The limit of detection was 10 IU/mL with a dynamic detection range of 10^2-10^9 IU/mL.

Rolling circle amplification

Rolling circle amplification (RCA) is a simple, reliable, and isothermal amplification method, which is driven by DNA polymerase to generate a long tandem repeat product based on a circular DNA template[54]. This technique does not require advanced laboratory equipment or experimental expertise. Compared with PCR, the main advantages of RCA include: (1) it is resistant to inhibitors present in clinical samples and requires almost no assay optimization[16]; and (2) it can amplify targets on solid support or in solution, offering opportunity for microarray and biosensor application[55] (Figure 3).

The property of a circular template for RCA makes it ideal for detection of HBV DNA, especially cccDNA. Margeridon *et al*[56] reported that cccDNA from liver biopsies could be amplified from as few as 13 copies using RCA. Martel *et al*[57] developed a method using *in vitro* completion/ligation of plus-strand HBV relax circular DNA and amplification using RCA. The method can amplify complete HBV genomes from serum with viral loads ranging from 10^3 to 10^8 IU/mL.

The isothermal amplification methods mentioned above offer several advantages over PCR, most importantly, they do not require an expensive and cost-intensive thermal cycler. Comparisons between isothermal amplification methods and conventional PCR are shown in Table 1.

Biosensors

Biosensors are analytical devices used for detection, which combine a biological component with a physicochemical detector[22]. Recently, an increasing number of biosensors have been used in clinical research; a common example is the blood glucose biosensor, which uses glucose oxidase to break blood glucose down[58,59]. Most of the clinical research on

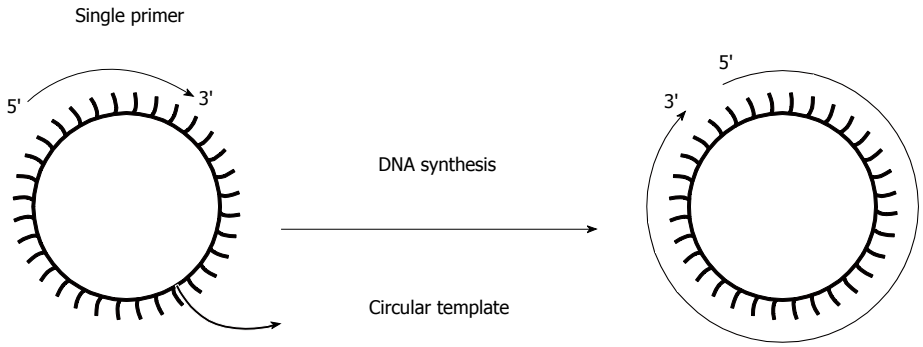

Figure 3 Scheme of the rolling circle amplification reaction. RCA rapidly synthesizes multiple copies of a single circular template with use of a single primer. RCA: Rolling circle amplification.

Table 1 Characteristics of isothermal amplification methods and polymerase chain reaction reviewed in this article

Method	Temperature requirement (℃)	No. of enzymes	Primer design	Product detection method	Rapid detection possibility	Tolerance to biological components
PCR	55-95	1	Simple	GE, ELISA, Real-time	Yes	No
IAM						
LAMP	60-65	1	Complex	GE, turbidity, Real-time	Yes	Yes
TMA	50-60	2	Simple	GE, ELISA, Real-time, ECL	Yes	No
NASBA	37-41	2 or 3	Simple	GE, ELISA, Real-time, ECL	Yes	No
RCA	37	1	Simple	GE, Real-time	Yes	No

ECL: Electrochemiluminescence; GE: Gel electrophoresis; PCR: Polymerase chain reaction; IAM: Isothermal amplification methods; LAMP: Loop-mediated isothermal amplification; TMA: Transcription-mediated amplification; NASBA: Nucleic acid sequence-based amplification; RCA: Rolling circle amplification.

biosensors was based on immunological reactions or DNA hybridization, and the biosensors always yielded rapid results with high sensitivity[60-62].

Electrochemical biosensors: Electrochemical biosensors work by detecting current or potential changes caused by binding reactions that occur on or near the electrode surface[63]. Recently, electrochemical biosensors have offered sensitivity, selectivity, and low-cost detection for DNA sequences and have attracted considerable attention. Ding *et al*[64] described a label-free electrochemical biosensor for the detection of oligonucleotides related to HBV sequences *via* the interactions of DNA with the redox-active complex 2,9-dimethyl-1,10-phenantroline cobalt[Co(dmp)(H_2O)(NO_3)$_2$]. The experiment was performed by hybridizing 21-mer DNA probes modified on glassy carbon electrode with target DNA and cobalt[Co(dmp)(H_2O)(NO_3)$_2$], whose sizes were comparable to the small groove of native double-helix DNA that was used as an electrochemical indicator. Under optimal conditions, the electrical signal had a linear relationship, with the concentration of target DNA ranging from 3.96×10^{-7} to 1.32×10^{-6} mol/L, and the detection limit was 1.94×10^{-8} mol/L.

Zhang *et al*[65] developed an electrochemical biosensor using diaquabis[N-(2-pyridinylmethyl)-benzamide-kappa N-2,O]-cadmium(Ⅱ) dinitrate as a new electroactive indicator for the detection of human HBV DNA. The hybridization between the probe and its complementary single-stranded DNA was determined by differential pulse voltammetry. Experiments with non-complementary oligonucleotides were carried out to assess the selectivity of the developed electrochemical DNA biosensor. HBV DNA could be quantified in a range from 1.01×10^{-8} to 1.62×10^{-6} mol/L with good linearity ($\gamma = 0.9962$). The detection limit was 7.19×10^{-9} mol/L.

Quartz crystal microbalance biosensors: Piezoelectric materials, typically crystals, generate an electrical potential in response to a mechanical force[19]. Piezoelectric biosensors are mass-sensitive, and the additional mass to the sensor causes a detectable change in the resonance frequency of the crystal. The most common type of piezoelectric biosensor is quartz crystal microbalance (QCM). QCM biosensors have gained increasing attention in recent years because of their high sensitivity, good specificity, low-cost, label-free detection, and rapid response[66].

Zhou *et al*[67] developed a highly sensitive piezoelectric HBV DNA biosensor based on the sensitive mass-transducing function of the QCM and the specificity of nucleic acid hybridization reaction. HBV nucleic acid probes were immobilized onto the gold electrodes of a 9 MHz AT-cut piezoelectric quartz crystal *via* the polyethyleneimine adhesion, glutaraldehyde cross-linking method or the physical adsorption method. The frequency shifts of hybridization have a good linear relationship with the amount of HBV DNA, when the amount was 0.02-0.14 mg/mL.

Peptide nucleic acid (PNA)-based piezoelectric

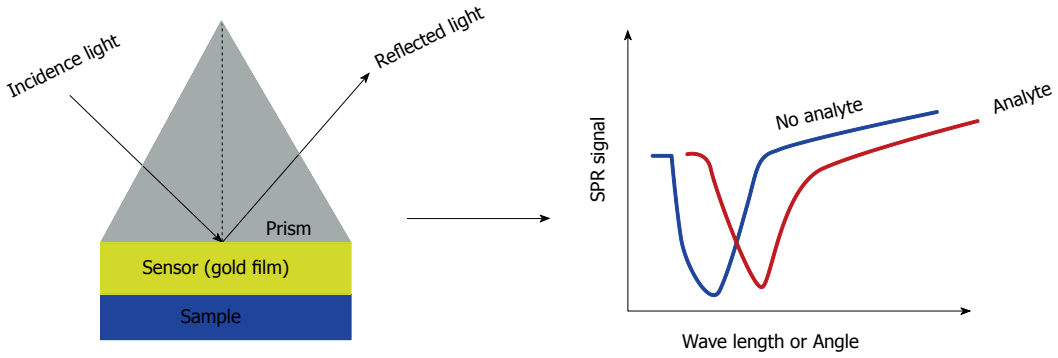

Figure 4 Scheme of the surface plasmon resonance biosensor. The incidence light causes the changes in wavelength or angle. Changes were detected in real time monitor. SPR: Surface plasmon resonance.

Table 2 Quantitative detection methods for hepatitis B virus discussed in this review

Method	Target	Detection range	Ref.
Architect	HBsAg	0.05-250.0 IU/mL	[12]
Elecsys	HBsAg	NA	[15]
UV spectrophotometry	HBV DNA	0.4-15000 ng/μL	[23]
In-house assays	HBV DNA	10^1-10^8 copies/reaction	[27]
based on real-time PCR	HBV DNA	50-10^8 IU/mL	[19]
Digital PCR	HBV DNA	Single copy	[39]
IAM			
LAMP	HBV DNA	48-10^8 IU/mL	[43]
TMA	HBV DNA	5×10^3-5×10^8 GE/mL	[47]
NASBA	HBV DNA	10^3-10^9 copies/mL	[52]
	HBV DNA	10^2-10^9 IU/mL	[53]
RCA	HBV DNA	10^3-10^8 IU/mL	[57]
Biosensors			
Electrochemical biosensors	HBV DNA	3.96×10^{-7}-1.32×10^{-6} mol/L	[65]
	HBV DNA	1.01×10^{-8}-1.62×10^{-6} mol/L	[66]
QCM biosensors	HBV DNA	0.02-0.14 mg/mL	[67]
	HBV DNA	8.6 pg/L[1]	[68]
Microcantilever biosensors	HBV DNA	23.1 fmol/L-2.31 nmol/L	[69]
SPR biosensors	HBV DNA	2 fg/mL[1]	[45]

[1]Only the limits of detection were given in these two papers. HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; NA: Not available; LAMP: Loop-mediated isothermal amplification; TMA: Transcription-mediated amplification; NASBA: Nucleic acid sequence-based amplification; RCA: Rolling circle amplification; QCM: Quartz crystal microbalance; SPR: Surface plasmon resonance; IAM: Isothermal amplification methods.

biosensor for real time monitoring of hybridization of HBV genomic DNA was constructed by Yao *et al*[68]. PNA probes can combine target sequences more effectively and specifically than DNA probes. The PNA probes were designed and immobilized on the surface of the biosensor to be a substitute for the conventional DNA probes for direct detection of HBV genomic DNA without previous amplification by PCR. The hybridization assay was completed in 50 min. The detection limit was 8.6 pg/L, and the clinical specificity was 94.44% in comparison with real-time PCR.

Microcantilever biosensors: In the last two decades, microcantilevers have emerged as a sensitive tool for the detection of chemicals and bio-organisms. Because of their light weight, small size, and high surface-to-volume ratio, microcantilever sensors improve the detection and identification of biological agents by several orders of magnitude[69-71]. An HBV DNA detection method using a silica-nanoparticle-enhanced dynamic microcantilever biosensor was developed by Cha *et al*[72], with a 243-mer nucleotide of HBV DNA pre-core/core region used as the target DNA. In this study, the capture probe immobilized on the microcantilever surface and the detection probe conjugated with silica nanoparticles were designed specifically for the target DNA. HBV DNA was detected using a silica-nanoparticle-enhanced microcantilever biosensor with a concentration of 23.1 fmol/L to 2.3 nmol/L, which was obtained from the PCR procedure. The HBV target DNA of 243-mer was detected up to the picomolar level without nanoparticle enhancement and up to the femtomolar level using a nanoparticle-based signal amplification process.

Surface plasmon resonance biosensors: Surface plasmon resonance (SPR) biosensors are optical sensors that exploit special electromagnetic waves, surface plasmon polaritons, to probe interactions between analytes and biomolecular recognition elements immobilized on the SPR sensor surface[73] (Figure 4). SPR biosensors are a label-free, real-time analytical technology for the detection of biological analytes and the analysis of biomolecular interactions.

Chuang *et al*[45] constructed a simple, low-cost, SPR-sensing cartridge based on the LAMP method for the on-site detection of HBV. The HBV template mixed in 10 μL LAMP solution could be detected by the new system in 17 min, even at the detection-limited concentration of 2 fg/mL. They also analyzed the correlation coefficients between the initial concentrations of HBV DNA templates and the system response at varying amplification times to establish an

optimum amplification time endpoint of 25 min (R^2 = 0.98).

All rapid and quantitative methods for HBV detection mentioned above are summarized in Table 2.

FUTURE PERSPECTIVES

The level of HBsAg has been suggested as a marker for the amount of cccDNA or infected liver mass. Although commercial assays are now available for HBsAg quantification, their dynamic range is narrow, and manual dilution is required if HBsAg concentration exceeds the dynamic range. More sensitive assays with broader detection ranges are needed for rapid and quantitative detection of HBV in clinical practice.

Our review describes the most assays that have been used for quantification of HBV DNA, along with their merits and limitations. PCR is the most widely used method in HBV DNA quantification, but it is also known to have erroneous results caused by its hybridization mechanism and false-negative results because of low HBV copy numbers. Additionally, PCR requires efficient control of thermal cycles and thus is instrument intensive. The development of isothermal amplification methods makes the amplification of HBV DNA without a thermal cycler possible, which also offers an opportunity for biosensor miniaturization. In spite of isothermal amplification and high sensitivity, isothermal amplification techniques have remained less utilized for developing commercial detection assays for HBV quantification. Biosensors have major advantages, such as speed, sensitivity, and low cost. Detection and quantification of viral components and viruses using biosensors is a new concept and is still in its infancy[63]. Tests developed previously are now being integrated into other novel platforms, such as the combination of LAMP and biosensors[45].

Development of rapid quantification detection for HBV is progressing towards procedural simplicity, tolerance to crude samples, high sensitivity, specific amplification, and robust reliable performance. With the development of technologies, quantification of HBV may be moved away from centralized laboratory facilities to other locations, such as emergency rooms, physician's office, and even the family. Point-of-care and point-of-patient testing may be the mainstream in the future[19,20].

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TOPIC HIGHLIGHT

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Early warning and clinical outcome prediction of acute-on-chronic hepatitis B liver failure

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Abstract

Hepatitis B virus (HBV) associated acute-on-chronic liver failure (ACLF) is an increasingly recognized fatal liver disease encompassing a severe acute exacerbation of liver function in patients with chronic hepatitis B (CHB). Despite the introduction of an artificial liver support system and antiviral therapy, the short-term prognosis of HBV-ACLF is still extremely poor unless emergency liver transplantation is performed. In such a situation, stopping or slowing the progression of CHB to ACLF at an early stage is the most effective way of reducing the morbidity and mortality of HBV-ACLF. It is well-known that the occurrence and progression of HBV-ACLF is associated with many factors, and the outcomes of HBV-ACLF patients can be significantly improved if timely and appropriate interventions are provided. In this review, we highlight recent developments in early warning and clinical outcome prediction in patients with HBV-ACLF and provide an outlook for future research in this field.

Key words: Chronic hepatitis B; Acute exacerbation; Acute-on-chronic liver failure; Early warning; Clinical outcome prediction

Core tip: Acute-on-chronic liver failure (ACLF) is a devastating disease with very high short-term mortality in chronic hepatitis B (CHB) patients, and acute exacerbation of CHB is an inevitable stage in the development of hepatitis B virus (HBV)-ACLF. The use of various prognostic models, careful observation of dynamic changes in certain clinical manifestations, biochemical variables, immune cells, cytokines, chemokines, HBV genotype, and mutants can also provide important references to help physicians determine the timely diagnosis and effective treatment

for patients with HBV-ACLF.

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INTRODUCTION

Despite the availability of potent vaccines, hepatitis B virus (HBV) infection remains a major public health problem worldwide with approximately 350 million individuals chronically infected[1-3]. Twenty percent of these chronically infected patients progress to life-threatening complications[4]. Acute-on-chronic liver failure (ACLF) is a critical complication of chronic hepatitis B (CHB) and can develop at any stage in the progression of CHB[5]. In China, it is estimated that approximately 70% of liver failure is caused by HBV infections, and, therefore, the prevalence of HBV-ACLF will likely increase in the coming decades.

HBV-ACLF has a poor prognosis, with an in-hospital mortality rate > 70% if emergency liver transplantation (LT) is not available. In the real-life setting, a very small number of HBV-ACLF patients undergo LT because of the shortage of liver donors. Although antiviral therapy can improve long-term survival and reduce the recurrence risk of ACLF, it does not improve short-term survival even when HBV DNA replication is controlled[6]. In this situation, it is believed that stopping or slowing the progression of CHB to ACLF at an early stage may be the most effective way of reducing the morbidity and mortality of patients with HBV-ACLF.

There is evidence suggesting that severe acute exacerbation of CHB is an inevitable stage in the development of ACLF, which is associated with many factors[7-9]. It can occur either over the natural course of the disease or following intensive chemotherapy or immunosuppressive therapy, and it easily progresses to ACLF if the abrupt flare is severe. In recent years, early warning and identification of severe acute exacerbation of CHB has attracted much attention, as effective control of the factors that trigger and worsen the disease is crucially important to slow down or reverse the progression of acute exacerbation of CHB to ACLF.

It is worth mentioning that due to the high cost of treatment and overly pessimistic view of the prognosis, many patients with HBV-ACLF choose to forego or discontinue treatment, especially in China. If an accurate assessment of the outcome of individual patients was possible, clinicians would encourage more active treatments for eligible patients with potentially better prognosis, and a considerable proportion of CHB patients may recover from HBV-ACLF.

In this review, we highlight recent developments in indicators and models for early warning and clinical outcome prediction of HBV-ACLF, which may help optimize current prevention and comprehensive treatments for ACLF in CHB patients.

CLINICAL MANIFESTATIONS FOR EARLY WARNING OF HBV-ACLF

Prior to the onset of typical abnormal laboratory findings, many patients present with certain clinical manifestations that may indicate the occurrence of ACLF in those with severe acute exacerbation of CHB[10]. In addition to the progressive symptoms of fatigue and weakness, it is also important to be aware of new and persistent nausea, vomiting, and hiccup in clinical practice, as these symptoms are related to the presence of endotoxemia. Although serum levels of total bilirubin (TBil) do not reach the diagnostic criteria for ACLF (10 times the upper limit of normal), rapid worsening jaundice also indicates a high probability of ACLF in CHB patients.

During CHB deterioration, manifestations such as aggravated bloating and hypoactive or missing bowel sounds are extremely common prior to or during the development of ACLF and are always associated with ascites, spontaneous bacterial peritonitis, or endotoxemia-induced toxic intestinal paralysis. For CHB patients with severe liver dysfunction, clinical manifestations such as skin petechiae and gingival bleeding are important signs of coagulation disorders. In addition, foul smelling gas and stools may indicate hepatic encephalopathy. Early diagnosis and treatment of coagulation disorders and hepatic encephalopathy may help to prevent or delay the development of ACLF.

However, it should be noted that the above-mentioned clinical manifestations are not specific to HBV-ACLF. To achieve more accurate identification of early warning, integrated monitoring and analysis of these clinical manifestations are warranted.

HOST PARAMETERS FOR EARLY WARNING AND PROGNOSIS PREDICTION IN HBV-ACLF

Biochemical indicators

It is well-known that liver dysfunction can lead to abnormal synthesis of important biologic signal molecules, metabolic disturbances, and alterations in serum biochemical indicators. Significantly elevated levels of alanine aminotransferase (ALT), TBil, and prothrombin activity (PTA) often suggest serious liver damage and are often used to diagnose HBV-ACLF[11]. However, there is no significant change in these indicators at the early stages of progression

from acute exacerbation of CHB to ACLF. Therefore, when only relying on the levels of these conventional biochemical indicators, it is difficult to achieve an early and accurate diagnosis of HBV-ACLF.

Evidence has shown that lipopolysaccharide (LPS)-induced endotoxemia can cause secondary hepatic injury, which is associated with the secretion of tumor necrosis factor α (TNFα) and the production of inflammatory cytokines[12,13]. In patients with ACLF, the highest serum levels of LPS are observed in the peak phase of TBil, and dynamic changes in LPS are correlated with disease severity[14]. Thus, for those patients with a high risk of endotoxemia, it is necessary to monitor serum LPS levels at admission and during treatment, as they will achieve maximum clinical benefit from timely and effective control of endotoxemia. Hyperammonemia is common in HBV-ACLF patients with hepatic encephalopathy and can cause problems with bilirubin metabolism by interfering with energy synthesis[15]. Kumar *et al*[16] recently reported that patients with persistent, mild hyperammonemia (≥ 85 μmol/L for 3 d) were more likely to develop complications and had higher mortality than those with serial ammonia levels < 85 μmol/L. Therefore, the evaluation of arterial ammonia at an early stage would be useful for risk stratification of disease progression.

Prealbumin is a protein produced by the liver, and it has a much shorter half-life (about 1.9 d) than that of albumin. A decline in prealbumin is more sensitive than albumin and more specific than ALT in reflecting early liver dysfunction. Huang *et al*[17] reported that there was also a positive correlation between the decline in prealbumin concentration and the severity of liver damage. Thus, monitoring the dynamic changes in serum prealbumin may provide early clues for the acute exacerbation of CHB to ACLB. Studies also reported that serum alpha fetoprotein (AFP) levels are increased in CHB patients with impaired liver functions[18,19], and compared to non-survivors with HBV-ACLF, survivors exhibited a higher increase in serum AFP level. However, a recent study showed that an early peak in AFP level prior to an elevation of prothrombin time may indicate a high risk of death[18]. Therefore, to predict the prognosis of HBV-ACLF, a comprehensive dynamic analysis of elevated AFP may be necessary.

It has been reported that serum metabolite and peptide profiling can vary during the progression of CHB to liver failure and that these dynamic changes may be used to distinguish different stages of the disease[20,21]. Recently, apolipoprotein (Apo) and lipid abnormalities were found to be associated with chronic liver failure[22]. In addition, serum levels of high-density lipoprotein and apolipoprotein A-I (ApoA-I) were inversely correlated with liver reserve and disease severity in cirrhotic patients with severe sepsis[23]. The low level of ApoA-I was also associated with a marked impairment in effective arterial volume, multiple organ dysfunction, and a poor prognosis[23]. Although the roles of ApoB, ApoE, and ApoA5 in ACLF have also been studied, the corresponding data are limited and future studies are needed.

In addition to the strong association between dysregulated iron homeostasis and both multi-organ failure and early mortality in ACLF[24], serum ferritin (SF) at admission was shown to be significantly higher in HBV-ACLF patients than in CHB and healthy controls, and elevated concentrations of SF were associated with increased severity of liver disease and 3 month mortality due to HBV-ACLF[25]. However, the predictive power of SF for mortality in HBV-ACLF patients was low [area under the curve (AUC) value: 0.640 ± 0.061]; and combining SF with the model of end-stage liver disease (MELD) score increased the power for predicting mortality (AUC value: 0.911 ± 0.035). Recently, sphingolipids were reported to be involved in the progression of liver disorders and to reflect the severity of hepatic injury in CHB[26]. The difference in sphingolipid profiles between CHB and HBV-ACLF patients was more significant than that between healthy controls and CHB, which indicated that serum sphingolipid levels were more likely to be associated with the progression of HBV-ACLF than CHB. For example, the serum levels of dhCeramides (dhCer) (d18:0/24:0) were significantly lower in patients who died compared with survivors. The decline in dhCer (d18:0/24:0) also showed a similar prognosis in terms of 3 mo mortality to that of the MELD score, thus, dhCer (d18:0/24:0) may be a useful prognostic biomarker for the early prediction of HBV-ACLF[27]. Additionally, the serum levels of thyroid stimulating hormone (TSH) have been reported to be a significant factor for predicting mortality in ACLF patients, and the cumulative survival rate decreased significantly when the serum TSH level was less than 0.38 IU/mL[28]. Therefore, serum TSH level may be a useful indicator for assessing severity and prognosis in ACLF patients.

It is known that kidney injury plays an important role in the prognosis of HBV-ACLF. Rapid deterioration of estimated glomerular filtration rate was recently reported to be associated with on-treatment mortality of CHB patients experiencing acute exacerbation[29]. In addition, Cystatin C (CysC) has been reported as a biomarker for predicting acute kidney injury in patients with ACLF[30]. By combining serum CysC and TBil, Wan *et al*[31] constructed a Prognostic Index (PI) to predict the 3 month mortality of HBV-ACLF patients [PI = 0.933 × CysC (mg/L) + 0.075 × TBil (mg/dL)]. Their findings showed that a PI < 3.91 may indicate an extremely good prognosis in patients with normal levels of serum creatinine (survival rate: 94.3% for PI < 3.9% *vs* 17.4% for PI = 3.91).

Immune cells and related cytokines

An abnormal immune reaction and an imbalance in the

production of proinflammatory and anti-inflammatory cytokines contribute to the outcome of acute exacerbation of CHB and prognosis of HBV-ACLF[32]. An abnormal immune reaction is mediated by the complex interactions between liver cells and host immune cells. Th17 cells are a subset of T helper cells that produce interleukin 17 (IL-17), thus promoting the activation of dendritic cells (DCs) and monocytes and enhancing the capacity to produce proinflammatory cytokines, including IL-1β, IL-6, TNFα, and IL-23[33]. It has now been confirmed that the overexpression of these proinflammatory cytokines plays an important role in liver damage progression[12,34] and that the activation of STAT3 upon IL-6 stimulation may contribute to the enhanced Th17 response (IL-17 production) in the deterioration of liver damage[35].

Some studies have reported that the frequency of peripheral Th17 cells and serum concentrations of IL-17 are gradually increased with immune inflammation aggravation in asymptomatic HBV carriers (AsC), CHB to ACLF, and overactive Th17 cells may be associated with immune tolerance breakthrough and severe exacerbation of CHB[33,36]. In addition, the frequency of peripheral Th17 cells in advanced-stage ACLF was significantly higher than that in early-stage ACLF, and surviving ACLF patients had an initially lower frequency of Th17 cells and IL-17 levels than non-survivors[36,37]. In ACLF patients, a positive correlation between peripheral Th17 cell frequency and both prothrombin activity and MELD score was observed[36]. Thus, monitoring of Th17 cell frequency and IL-17 levels helps to assess the exacerbation of liver damage during chronic HBV infection[33], and high Th17 cell frequency and serum IL-17 concentration in ACLF patients may indicate a poor prognosis.

It is well-known that regulatory T (Treg) cells can suppress the expansion and interferon gamma (IFNγ) secretion of autologous peripheral blood mononuclear cells (PBMCs) when stimulated with HBV antigen. Recently, Th17/Treg cell imbalance in disease progression was also demonstrated. For example, in the remission stage of ACLF, Th17 cells increase and Treg cells decrease, creating an imbalance that is negatively correlated with disease progression[38]. Thus, the ratio of Th17/Treg cells may be a good prognostic marker in predicting ACLF progression[37], and restoring the Th17/Treg cell ratio could maintain the immune system at a steady state in ACLF patients.

DCs are specific antigen-presenting cells (APCs) and are abundant in the liver. The number and function of DCs are closely associated with the prognosis of HBV-ACLF patients treated with different therapies. A high number of myeloid DCs at baseline and the recovery of myeloid DCs numbers at the end of treatment may represent a prognostic marker for a favorable response to corticosteroids and granulocyte colony-stimulating factor (G-CSF) in patients with HBV-ACLF[39,40].

It is well-known that the number of monocytes and macrophages is greatly increased in the circulation and liver of patients with ACLF[41]. Activated monocytes/macrophages release a large number of proinflammatory and anti-inflammatory cytokines, and the level of proinflammatory cytokine production is closely related to disease severity in patients with liver failure[12]. The procoagulant molecule, human fibrinogen-like protein 2/fibroleukin (hfgl2), is an inflammatory mediator produced by activated macrophages and can directly cleave prothrombin into activated thrombin, resulting in intravascular fibrin deposition in the liver[42]. Importantly, there is a positive correlation between hfgl2 expression and the severity of liver damage in CHB patients[43]. Compared to 7.7% of CHB patients with mild liver damage expressing hfgl2, 91.3% of HBV-ACLF patients had hfgl2 expression[43], and high levels of virus-induced hfgl2 were observed in liver sections from patients with HBV-ACLF[44]. Thus, hfgl2 may play a pivotal role in initiating acute severe hepatitis in those with chronic hepatitis, and hfgl2 expression in peripheral blood may also be used as a biomarker to monitor the acute exacerbation of CHB and to assess the severity of ACLF.

In addition to the secretion of inflammatory cytokines, monocytes/macrophages can induce adaptive immune responses through their antigen-presenting functions. The expression of human leukocyte antigen (HLA) class Ⅱ molecules, especially HLA-DR (a heterodimeric cell surface glycoprotein) is particularly important for monocyte activation. Antoniades *et al*[45] recently reported a strong relationship between monocyte HLA-DR expression and the indices of disease severity, mediators of inflammation, and outcome. A level of HLA-DR ⩽ 15% had 96% sensitivity, 100% specificity, and 98% accuracy in predicting poor prognosis in patients with liver failure[45]. Xing *et al*[46] also reported similar findings, suggesting that monocyte HLA-DR expression in patients who died was significantly lower than that in patients who survived in the early and late stages of ACLF. Therefore, the functional status of PBMC and HLA-DR expression may be used to monitor disease severity and predict clinical outcome in patients with ACLF.

Chemokines

The CC chemokines, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-3α (MIP-3α), may be involved in the pathogenesis of ACLF. Compared to healthy controls and patients with chronic hepatic failure, serum concentrations of MCP-1 and MIP-3α were significantly enhanced in ACLF patients[47]. Interestingly, Leifeld *et al*[48] investigated the time course of CC-chemokine release in concanavalin A and LPS-induced liver failure mouse models and found that intrahepatic MCP-1 and MIP-3α upregulation occurred prior to hepatic infiltration and liver damage. In addition, elevations

in MCP-1 and MIP-3α serum concentrations reflected the degree of liver inflammation[49]. Therefore, serum concentrations of MCP-1 and MIP-3α may be used as early warning indicators of ACLF.

In endotoxemic liver injury in mice, CXC chemokines are instrumental in regulating endotoxin-induced transmigration and extravascular tissue accumulation of leukocytes, and interference with MIP-2 and neutrophil chemoattractant functions protect against septic liver damage[50]. In massive hepatectomy induced acute liver damage, the CXC chemokine CXCL1 was significantly increased[51]. Additionally, the serum level of CXCL10 was significantly related to the degree of liver inflammation or damage in CHB patients, and it may play an important role in trafficking inflammatory cells to the local focus in the liver and induce disease progression[15]. It is worth mentioning that the polymorphism G-201A is present in the promoter of CXCL10 gene and can alter the binding affinity of nuclear protein and regulate CXCL10 expression. Therefore, G-201A may be involved in the genetic variation underlying the susceptibility of individuals to acute exacerbation of CHB[52].

Host hemodynamic derangements

Recently, Garg *et al*[53] reported that the presence of a high hepatic venous pressure gradient (HVPG) is an independent baseline predictor of mortality in HBV-ACLF patients, as the raised portal pressure predisposes patients to a high risk of variceal bleeding. During the dynamic follow-up of surviving patients, these authors observed a reduction in HVPG; and this reduction correlated with clinical and biochemical recovery and a reduction in Child-Turcotte-Pugh (CTP) score. It is worth mentioning that hyponatremia is also common in patients with ACLF. For example, compared to 12.3% of patients without ACLF in the CANONIC study, 24.3% of patients with ACLF had hyponatremia at inclusion[54]. In addition, the presence of hyponatremia influences the outcome of patients with ACLF. The 3 mo transplant-free survival in ACLF patients with hyponatremia is only 35.8% but is as high as 58.7% in patients without hyponatremia[54]. Thus, hepatic and systemic hemodynamic derangements may predict early mortality and recovery in patients with ACLF.

VIRAL FACTORS IN THE EARLY WARNING OF HBV-ACLF

It is well-known that ACLF patients have distinct quasi-species characteristics with higher complexity and diversity within the basal core promoter (BCP)/precore (PC) region of HBV[55]. Both single mutations, including T1753C, A1762T, G1764A, A1846T, C1913A/G, G1896A, and G1899A, and double mutations, including A1762T/G1764A and G1896A/G1899A, are more frequently detected in HBV-ACLF patients than in CHB patients[56,57]. At present, there is evidence suggesting that CHB patients infected with BCP/PC mutations are more susceptible to ACLF, and HBV-ACLF patients with BCP/PC mutations also have a higher risk of mortality than those infected with BCP/PC wild-type virus[58-60].

It should be noted that either the absolute frequency of genotype B or the ratio of genotype B to C is significantly higher in HBV-ACLF patients than in CHB patients[58,61]. In genotype B patients, the A1762T/G1764A, A1846T, and G1896A mutations are also significantly more prevalent in patients with ACLF than in patients with CHB, and genotype B patients with G1896A and A1762T/G1764A have a higher tendency to develop HBV-ACLF than patients with genotype C[62]. To a certain extent, HBV genotyping and detection of BCP/PC mutations may have implications for the prediction of HBV-ACLF in clinical practice.

Studies have also shown that severe HBV reactivation can lead to disease flare and even liver failure, which often occurs following interrupted/discontinued antiviral therapy, intensive chemotherapy, or immunosuppressive therapy. For those who are at high risk of HBV reactivation, dynamic monitoring of serum HBV DNA would help in the early warning of possible HBV-ACLF occurrence, and long-term control of viral replication is necessary and important to prevent the occurrence of ACLF. Additionally, evidence suggests that effective control of HBV DNA replication would help to reduce the long-term mortality and recurrence rates of HBV-ACLF[63].

SCORING SYSTEMS FOR PROGNOSIS PREDICTION IN HBV-ACLF

In the past decades, a number of scoring systems have been used for outcome prediction in end-stage liver diseases, including CTP, MELD, and their derivative scores, King's College Hospital (KCH) criteria, sequential organ failure assessment (SOFA), acute physiology, and chronic health evaluation (APACHE), Clichy criteria, and artificial neural network (ANN). However, not all of these scores are well validated in HBV-ACLF patients. Consequently, many new mathematical models have recently been developed using multiple regression and have shown certain advantages.

The CTP score was originally devised for the assessment of liver disease severity to predict the outcome of patients with cirrhosis, in whom surgical treatment for portal hypertension was planned. The CTP score mainly applies to cirrhotic patients, with subjective judgments on hepatic encephalopathy and ascites and limited discriminant ability. Additionally, the CTP score does not distinguish the clinical significance between mild-to-moderate and severe abnormal laboratory parameters, and patients with different disease severity have the same CTP score[64]. Thus, the CTP score has poor accuracy in predicting

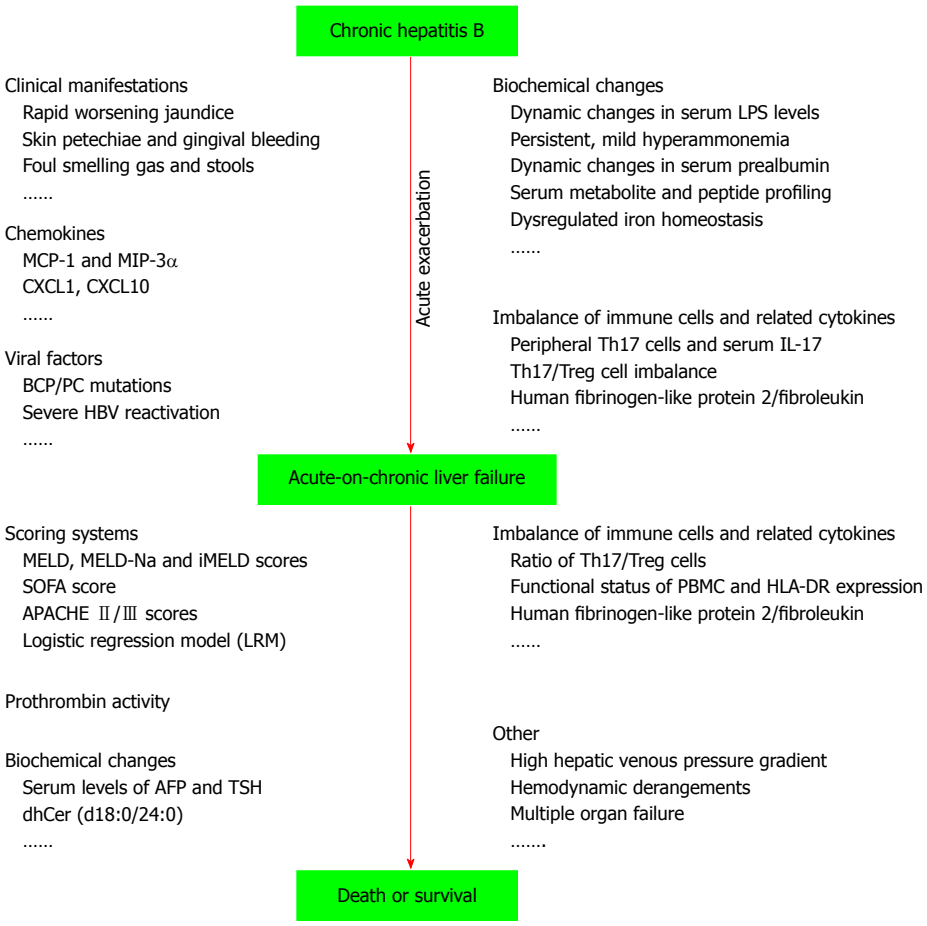

Figure 1 Overview of indicators and models for early warning of the progression of acute exacerbation of chronic hepatitis B to acute-on-chronic liver failure and prediction of the outcome of patients with hepatitis B virus-acute-on-chronic liver failure. LPS: Lipopolysaccharide; MCP-1: Monocyte chemoattractant protein-1; MIP-3α: Macrophage inflammatory protein-3α; MELD: Model of end-stage liver disease; BCP: Basal core promoter; PC: Precore; HBV: Hepatitis B virus; SOFA: Sequential organ failure assessment; APACHE: Acute physiology and chronic health evaluation; AFP: Alpha-fetoprotein; TSH: Thyroid stimulating hormone.

the prognosis of ACLF patients. Similarly, the KCH and Clichy criteria are also rarely used to predict the prognosis of patients with ACLF. Instead, they are more appropriate for assessing the severity of patients with acute liver failure and the urgency for LT.

In order to overcome these limitations of the CTP, the MELD score was developed, and its advantages are that it can accurately reflect hyperbilirubinemia, coagulation disorders, and renal impairment. Many reports have confirmed that the MELD score is a good mortality predictor in ACLF patients awaiting LT. However, there is some controversy regarding its efficacy in predicting post-transplantation outcomes[65]. For example, ACLF patients with a high MELD score (≥ 30) have a good post-transplantation outcome compared with those patients with a score < 30 after LT[66-68]. Therefore, the MELD score may not be a good prognostic scoring system for HBV-ACLF patients undergoing LT.

The MELD-based MELD-Na and iMELD scores were also investigated in clinical practice[69], but there was no significant improvement in prediction accuracy of the prognosis of HBV-ACLF. This is because important factors (*i.e.*, hepatic encephalopathy, hepatorenal syndrome, and upper gastrointestinal bleeding) that can affect the prognosis of patients were not taken into consideration in these scores[70-72]. In addition, diuretic therapy or artificial liver treatment can easily lead to a fluctuation in serum Na, bilirubin, and creatinine, and the latter could significantly affect the reliability of MELD-associated scores. Noticeably, MELD-based scores are still mainly used in cirrhotic patients. For non-cirrhotic HBV-ACLF patients, their prediction efficacy is relatively poor. In a previous study, we evaluated the efficacy of the MELD and MELD-Na scores in predicting short-term prognosis of HBV-ACLF patients, and the results showed that both the MELD (AUC: 0.758 *vs* 0.840) and MELD-Na (AUC: 0.776 *vs* 0.849) scores appeared to have a weaker predictive effect in non-cirrhotic HBV-ACLF patients than in cirrhotic HBV-ACLF patients[73]. Therefore, for non-cirrhotic HBV-ACLF patients, care should be taken when MELD-based scores are used to predict possible outcomes.

For a long time, the SOFA score and APACHE Ⅱ/Ⅲ scores were reported to be good indicators of

prognosis in critically ill patients. Except for initial scores of more than 11 (mortality rate > 90%), a decreasing SOFA score during the first 48 h predicts a mortality rate of at least 50% in intensive care unit patients[74]. In patients with alcohol-related ACLF, the APACHE Ⅱ score seems superior to SOFA, CTP, and MELD in predicting short-term mortality[75]. However, the use of SOFA and APACHE Ⅱ/Ⅲ scores in patients with HBV-ACLF is rare. Thus, it is unclear whether these scores could effectively predict the outcome of patients with HBV-ACLF.

In recent years, many new mathematical models have been established to assess the short-term prognosis of HBV-ACLF patients[76]. For example, the logistic regression model established by Zheng *et al*[70] has greater accuracy than MELD and CTP in predicting the prognosis of HBV-ACLF patients, regardless of the presence or absence of cirrhosis. The predictive validity of the APLH-Q score established by cox proportional hazard regression analysis seems significantly better than that of the previously reported LSM and MELD[77]. In addition, Ning *et al* have also established a Tongji prognostic predictor model (TPPM) by integrating biochemical parameters, coagulation parameters, indicators of hepatitis virology, and complications. The TPPM has better sensitivity and specificity in predicting 3 mo mortality in HBV-ACLF patients than the MELD score. However, these mathematical models were used in single-center retrospective studies with a relatively small sample size and need to be validated in other well-designed prospective studies.

CONCLUSION

ACLF is a devastating disease with a very high short-term mortality in CHB patients, and acute exacerbation of CHB is an inevitable stage in the development of HBV-ACLF. Here, we reviewed the indicators and models for early warning of the progression of acute exacerbation of CHB to ACLF and the prediction of the outcome of patients with HBV-ACLF (Figure 1). Careful observation of dynamic changes in certain clinical manifestations and laboratory variables (such as host biochemical variables, immune cells, cytokines, chemokines, virus genotypes, and mutants) can provide important references to help physicians determine the timely diagnosis and effective treatment of HBV-ACLF. However, due to the complexity of HBV-ACLF development, a considerable number of those indicators and models have either methodological or reporting limitations. For example, their accuracy in early warning or outcome prediction of HBV-ACLF may vary significantly between cirrhotic and non-cirrhotic patients. Thus, ideal early warning and prognostic prediction systems, which pay more attention to various underlying diseases and complications, are needed. In addition, their accuracy and validity should be verified in well-designed prospective studies with a large sample size.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis C virus

Hepatitis C virus-associated neurocognitive and neuropsychiatric disorders: Advances in 2015

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Abstract

Since its identification in 1989, hepatitis C virus (HCV) has emerged as a worldwide health problem with roughly 185 million chronic infections, representing individuals at high risk of developing cirrhosis and liver cancer. In addition to being a frequent cause of morbidity and mortality due to liver disease, HCV has emerged as an important trigger of lymphoproliferative disorders, owing to its lymphotropism, and of a wide spectrum of extra-hepatic manifestations (HCV-EHMs) affecting different organ systems. The most frequently observed HCV-EHMs include mixed cryoglobulinemia and cryoglobulinemic vasculitis, B-cell non-Hodgkin's lymphoma, nephropathies, thyreopathies, type 2 diabetes mellitus, cardiovascular diseases, and several neurological conditions. In addition, neuropsychiatric disorders and neurocognitive dysfunction are reported in nearly 50% of patients with chronic HCV infection, which are independent of the severity of liver disease or HCV replication rates. Fatigue, sleep disturbance, depression and reduced quality of life are commonly associated with neurocognitive alterations in patients with non-cirrhotic chronic HCV infection, regardless of the stage of liver fibrosis and the infecting genotype. These manifestations, which are the topic of this review, typically occur in the absence of structural brain damage or signal abnormalities on conventional brain magnetic resonance imaging (MRI), although metabolic and microstructural changes can be detected by *in vivo* proton magnetic resonance spectroscopy, perfusion-weighted and diffusion tensor

MRI, and neurophysiological tests of cognitive processing. Several lines of evidence, including comparative and longitudinal neuropsychological assessments in patients achieving spontaneous or treatment-induced viral clearance, support a major pathogenic role for HCV in neuropsychiatric and neurocognitive disorders.

Key words: Extra-hepatic manifestations; Hepatitis C virus syndrome; Hepatitis C virus; Neurocognitive impairment; Neuropsychiatric disorders; Proton magnetic resonance spectroscopy; Sleep disorder

Core tip: Neurocognitive dysfunction, sleep disturbance, depression, fatigue and reduced quality of life are common manifestations of chronic hepatitis C virus (HCV) infection. Neuropsychological performance is impaired in HCV patients, in the absence of structural brain alterations on conventional magnetic resonance imaging (MRI). Brain metabolic and microstructural changes are easily detected by *in vivo* proton magnetic resonance spectroscopy and perfusion-weighted/diffusion tensor MRI, enabling detection of brain dysfunction in clinically asymptomatic subjects. The regional distribution of metabolic changes indicates an exclusive involvement of telencephalic areas, but not the diencephalon or brainstem. HCV is likely to play a major pathogenic role in these disorders.

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INTRODUCTION

Previously designated as non-A non-B hepatitis, the hepatitis C virus (HCV) was unequivocally recognized and established as a distinct virus after its isolation in 1989[1]. In the following 26 years, HCV has been the subject of extensive research which has expanded our knowledge of the biomolecular and clinical features of HCV infection tremendously, and has paved the way for the introduction of newly synthesized, highly effective, direct-acting antiviral agents that are making eradication of the virus an achievable goal in nearly all chronically infected patients.

Acute HCV infection is usually asymptomatic and able to resolve spontaneously in as high as 90% of cases, and thus remains undiagnosed in the majority of patients. Even so, progression to chronic infection occurs frequently. Thus, HCV represents a worldwide health problem considering that at least 3% of the world population, corresponding to roughly 185 million people, is chronically infected. However, infection rates are highly variable across different geographic areas[2]. Of the six major HCV genotypes (gt), gt-1 is the most common worldwide, with subtype 1b being prevalent in Europe and subtype 1a in the United States. In intravenous drug users, gt-3a is more frequently detected in Europe and gt-2 in the Mediterranean area. Genotypes 5 and 6 are rare, whereas gt-4 occurs in over 20% of the infected Egyptians.

Chronic HCV infection progresses to liver cirrhosis in 10% to 40% of the patients (this condition is the leading indication for liver transplantation) and liver cancer (*i.e.,* hepatocellular carcinoma, HCC) in 1% to 5% per year. The annual mortality rate has been calculated to be roughly 4% of patients with cirrhosis and 30% of those with HCC.

HCV-related neurological dysfunction is frequently observed either at cirrhotic or pre-cirrhotic stages. A major central nervous system (CNS) complication observed in patients with cirrhosis of differing aetiology, including those with HCV infection, is hepatic encephalopathy (HE), a potentially reversible neuropsychiatric syndrome, with symptoms ranging from clinically undetectable neuropsychological disturbances (minimal HE) to severe impairment of attention and arousal (overt HE). In addition, a large number of neurological complications occur in patients with chronic HCV infection independently of liver disease, including metabolic, inflammatory and autoimmune conditions affecting the CNS, as well as the peripheral nervous system and muscles[3,4].

CONCEPT OF HCV SYNDROME

HCV has been confirmed as both a hepatotropic and lymphotropic virus. Consequently, the liver is not the only target of HCV infection, so that several other organs and tissues are also likely to be involved[5].

The term "HCV syndrome" was coined to include both hepatic and extra-hepatic manifestations of HCV infection[6,7]. One major extra-hepatic disorder associated with HCV is mixed cryoglobulinemia, which, in a minority of patients, results in clinically overt cryoglobulinemic vasculitis (CV)[8]. In agreement with the experience of other research groups, our data indicate that 90% or more of CV patients are in fact anti-HCV/HCV RNA positive. Figure 1 summarizes the major extra-hepatic manifestations that have been diagnosed, based on our own experience[9], in chronically infected HCV patients, with or without mixed cryoglobulinemia. As the figure illustrates, the most convincing associations of chronic HCV infection are with subsets of B-cell non-Hodgkin lymphoma (B-NHL), membranous or membrano-proliferative glomerulonephritis, IgM monoclonal gammopathy of undetermined significance (MGUS), peripheral neuropathy, and CNS disorders.

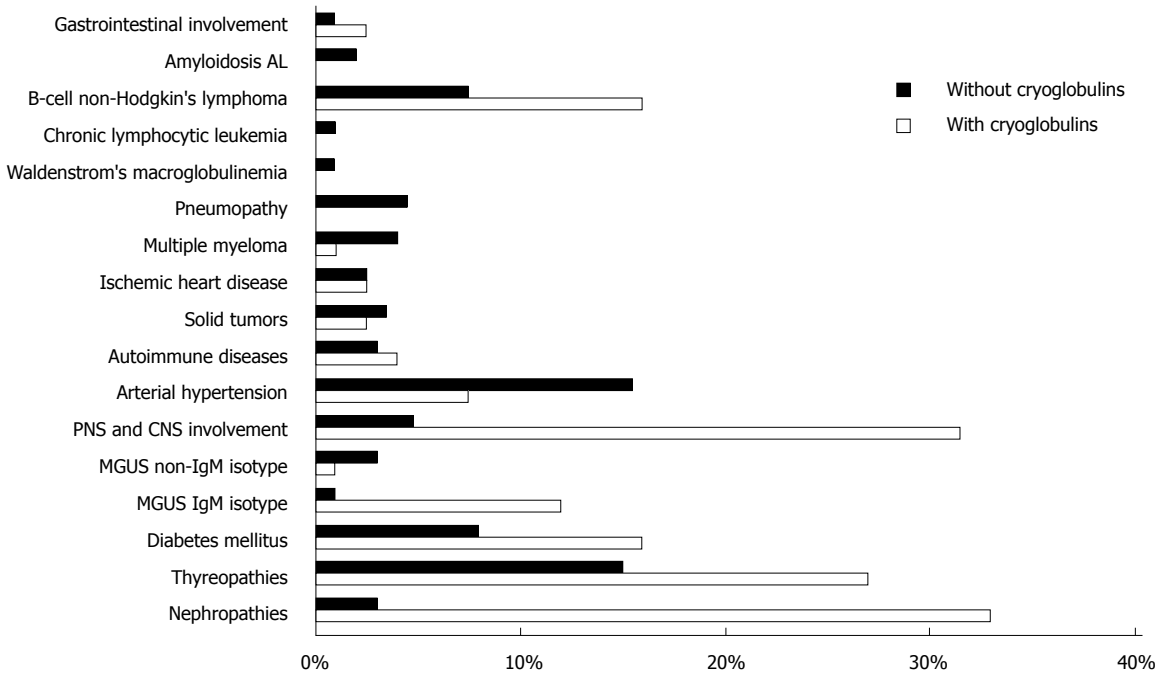

Figure 1 Summary of major extra-hepatic manifestations observed in patients with chronic hepatitis C virus infection. Percentages of patients are represented in graphic form. The presence or absence of cryoglobulinemia is indicated by white and black, respectively. The most convincing associations of chronic hepatitis C virus (HCV) infection are with subsets of B-cell non-Hodgkin lymphoma (B-NHL), membranous or membrano-proliferative glomerulonephritis, IgM monoclonal gammopathy of undetermined significance (MGUS), peripheral neuropathy, and central nervous system (CNS) disorders.

PATHOGENETIC IMPLICATIONS

The role played by HCV in the pathogenesis of the largely autoimmune and neoplastic diseases remains undefined, but a reasonable hypothesis is that HCV infection is an important component of a polyfactorial process in which multiple genetic and environmental cofactors are involved to a variable extent. The long-term observation of cohorts of HCV-positive patients indicates that many of them develop a complex and progressively more severe spectrum of HCV-related disorders, advancing from mild and often limited features, to systemic and more severe complications that may include HCC, B-NHL and possibly other neoplastic histotypes[10].

Studies of the biology of HCV have been hampered by the fact that for many years chimpanzees were the only non-human *in vivo* models, and there was an unmet need for the development of *in vitro* experimental models. More recently though, researchers determined how to grow infectious HCV particles in cell cultures, which has resulted in a better understanding of the virus-host interactions enhancing virus propagation. Furthermore, small animal models permissive to HCV have been established, which now make it possible to investigate new antiviral agents and in the future, potential vaccine preparations[11].

Recognized pathogenetic mechanisms in several HCV-related neurological disorders have in common an upregulated host immune response accompanied by production of autoantibodies, immune complexes, and cryoglobulins. However, alternative mechanisms have been proposed for the neurocognitive impairment and neuropsychological changes observed in HCV patients. The isolation of HCV RNA in microglial cells and astrocytes and evidence that brain endothelial cells are permissive for HCV entry and replication suggest an independent life and a pathogenic role of the virus in the brain[12]. These findings are at variance with the detection of nonreplicative HCV RNA in epineurial macrophages, but not in the "endoneurial microglia", of nerve biopsies of patients with HCV infection and peripheral neuropathy[13,14].

Although the clinical spectrum of HCV syndrome has been expanded, the exact pathophysiological mechanisms of cognitive impairment, neuropsychiatric disorders, and sleep disturbance remain poorly understood. Efforts to elucidate molecular pathways have provided results suggesting a pathogenic role for the virus itself, iatrogenic factors, and inflammatory cytokines in distinct neuropsychiatric conditions. Therefore, the detection of defective central serotoninergic and dopaminergic neurotransmission in some HCV patients with neuropsychiatric symptoms and mild or no liver disease has suggested a possible role for HCV in inducing dysfunction in selective aminergic systems[15]. On the other hand, in patients under treatment with interferon (IFN), the occurrence of depression has been correlated with depletion of platelet serotonin, an effect also expected within the CNS, due to the effectiveness of antidepressant drugs that inhibit serotonin reuptake[16,17]. Other proposed mechanisms for dysfunction of the CNS and the neuroendocrine system include cytotoxic effects

induced by cytokines released during systemic or brain immune activation[18,19].

COGNITIVE IMPAIRMENT

Altered neuropsychological performance and neurocognitive impairment are frequently reported in patients with chronic HCV infection, often at stages characterized as having a lack of significant liver fibrosis and cirrhosis. These alterations typically occur independently of HCV genotype and in the absence of structural brain damage or signal abnormalities on conventional brain magnetic resonance imaging (MRI). Over the last few years, a number of factors, including associated comorbidities, alcohol misuse, substance abuse, interferon treatment, and HCV itself, have been investigated to assess their role as deteriorating or causative contributors to HCV-associated neurocognitive disorder (HCV-AND). Available data suggest that HCV-AND is unrelated to advanced liver disease, and therefore, the nature of this condition is distinct from the potentially reversible neuropsychological/neurophysiological complications observed in individuals with minimal HE, *i.e.*, with biopsy-proven compensated cirrhosis or portal-systemic encephalopathy. Unravelling the contribution of different factors to cognitive dysfunction and elucidating the pathogenic role of HCV represent issues of major importance for making personalized treatment options available. Emerging lines of evidence suggest that the profile of neuropsychological dysfunction in HCV-infected patients is characterized by impairment in executive function, sustained attention, working memory, verbal learning and verbal recall. Conversely, the neuropsychological complex observed in minimal HE includes impairment in psychomotor speed, selective attention, visuoconstructive function and executive function. Forton *et al*[20], using *in vivo* proton magnetic resonance spectroscopy (^{1}H MRS), first reported elevated choline/creatine ratios in basal ganglia and frontal white matter (WM) of HCV-infected patients with mild liver disease, but not in patients with hepatitis B virus (HBV) infection. Subsequently, these alterations were correlated with impaired concentration and working memory in a group of patients with active viral replication[21], whereas the cognitive performance of patients who had achieved HCV clearance was equivalent to that of controls. Intriguingly, metabolic changes detected by ^{1}H MRS in HCV patients were different from abnormalities previously associated with minimal HE, which were characterized by reduced choline/creatine ratios[22]. The occurrence of significant deficits in executive function and attention, with worse performance in patients complaining of fatigue, has been reported in HCV patients with normal liver function. This neuropsychological profile was correlated with a decrease in N-acetylaspartate (NAA)/creatine ratios in the cerebral cortex[23]. Conversely, only slight attention deficit and impairment of verbal learning were documented in a study involving 37 HCV patients without significant liver disease or other relevant comorbidities. In these individuals, ^{1}H MRS revealed increases in choline and decreases in NAA in the centrum semiovale, as well as increases in creatine, a marker of astrocytic gliosis and microglia activation, in basal ganglia[24]. Increased levels of creatine were also detected in the basal ganglia of HCV patients with impaired attention and working memory in another study; unexpectedly, increased concentrations of NAA plus N-acetyl-aspartyl-glutamate, possible markers of on-going repair and neuroprotective mechanisms, were also found in these patients[25]. More recently, decreases in NAA/creatine ratios in the fronto-parietal WM, in the absence of other metabolic changes, were found in 15 patients with mild liver disease, diminished visual concentration endurance and unaffected executive function[26]. These alterations were coupled with increased perfusion of basal ganglia and reduced perfusion of left frontal cortex, bilateral temporoparietal cortices, and posterior cingulate gyrus[27]. Furthermore, increased myoinositol in bilateral frontal WM and decreased levels of NAA in parietal WM without changes in basal ganglia were documented in a cohort of HCV treatment-naïve patients with reduced psychomotor speed and verbal fluency. These patients also showed microstructural brain pathology in the striatum, external capsule, and fronto-occipital fasciculus by diffusion tensor MRI[28]. Taken together, the variability observed across different studies likely reflects the use of different psychometric scales and heterogeneity among recruited patients.

Results of studies addressing the quantification of brain metabolite concentrations in HCV patients with neuropsychological and neurocognitive dysfunction are shown in Figure 2. Despite a number of limitations, these studies demonstrate that chronic HCV infection is associated with significant brain metabolic alterations as a result of activation and proliferation of glial cells (including astrocytes, microglia, and monocyte/macrophages) and increased turnover of myelin membranes. The regional distribution of ^{1}H MRS abnormalities suggests that only cortical and subcortical telencephalic areas, but not the thalamus or posterior fossa structures, are involved in HCV-AND. These findings are in agreement with results of neuropathological studies in human immunodeficiency virus (HIV)-HCV co-infected individuals, demonstrating the presence of HCV RNA in the frontal cortex, basal ganglia, and subcortical white matter, but not in the thalamus, cerebellum, and brainstem. In addition, HCV NS5A and NS3 proteins, as well as the core antigen, were found in astrocytes and macrophage/microglial cells[29]. These findings are relevant, in view of the evidence that the core protein may trigger and support a neurodegenerative cascade through the activation of ERK/STAT3 pathway[30]. Studies using ligands that bind serotonin and dopamine transporters have revealed a decreased density of serotonin

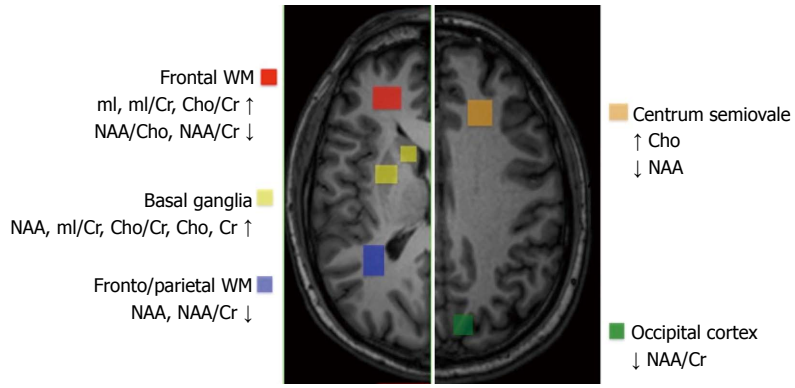

Figure 2 Quantification and localization of brain metabolite concentrations in hepatitis C virus patients exhibiting neuropsychological and neurocognitive dysfunction. The regional distribution of proton magnetic resonance spectroscopy (^{1}H MRS) abnormalities suggests that only cortical and subcortical telencephalic areas, but not the thalamus or posterior fossa structures, are involved in hepatitis C virus (HCV)-associated neurocognitive disorder (HCV-AND).

and dopamine nerve terminals on single-photon-emission computed tomography in HCV patients with neuropsychiatric disorders and cognitive impairment, even in the absence of significant liver disease. Moreover, images obtained through positron-emission tomography with the use of fluorodeoxyglucose revealed decreased glucose metabolism in limbic, frontal, parietal and temporal cortices[15,31,32]. Earlier studies, employing neurophysiological tests of cognitive processing, revealed delayed peak latencies and reduced amplitudes of P300 in HCV patients compared to healthy subjects, thus providing a potential and sensitive neurophysiological measure of subclinical cognitive impairment[33].

The characterization of neuropsychological/cognitive dysfunction in HCV patients has been the subject of several studies. Defects in verbal recall and working memory were found in one-third of HCV patients, including individuals with advanced liver fibrosis, cirrhosis, and a history of alcohol misuse and/or intravenous drug abuse. In contrast to earlier reports, cognitive performance was not affected by the degree of fibrosis[34]. Impairment of verbal learning and memory was detected in a cohort of HCV and HBV patients, including patients with cirrhosis, as compared to healthy controls[35], whereas verbal learning, auditory attention, and reasoning/mental flexibility were affected in a cohort of 24 non-cirrhotic HCV patients with a negative history for medical/psychiatric comorbidities, and drug and/or alcohol abuse[36]. More recently, the study of a small and well-selected cohort of homogeneous state-infected, treatment-naïve patients with mild liver involvement and a similar history of iatrogenic HCV exposure, showed that half of HCV RNA-positive patients had alterations in memory, sustained attention, and delayed auditory recognition, as compared to HCV RNA-negative subjects who had spontaneous viral clearance[37]. The effect of successful anti-HCV treatment on neurocognitive measures was recently investigated in a longitudinal multicentre study involving a relatively large number of HCV patients, 15% of whom had cirrhosis. Long-term evaluation at 12 mo or more following treatment showed a significant improvement in vigilance and working memory in patients who had achieved sustained virological response (SVR), but not in non-responders[38].

PSYCHIATRIC DISTURBANCES

Sexual dysfunction, emotional distress and psychiatric disturbances are frequently described in HCV-infected patients. People with HCV infection have a higher risk for depression, anxiety, somatization, compulsiveness, insecurity, aggression/hostility, phobic anxiety, and psychosis[39]. Although major psychiatric co-morbidities, concomitant or previous substance abuse, and the psychological consequences of having a chronic life-threatening illness may account for most of these manifestations, several studies have indicated a primary role for HCV. Based on DSM-IV criteria, 28% of chronically HCV-infected individuals experience depression, which makes it difficult for them to adhere to therapy[40,41]. Major depressive disorder[42] and recurrent brief episodes of depression unrelated to interferon treatment have been reported[43]. Nevertheless, the prevalence of psychiatric symptoms is probably underestimated in routine clinical examination[44], even though psychiatric screening, accurate diagnosis, and confirmatory evaluation by validated measures are strictly required[45]. Depression, anxiety and fatigue are among the most common adverse events in IFN/ribavirin treatment, and their cumulative incidence seems to increase during IFN treatment, but returns to baseline values following completion of therapy[46].

No major risk factors for treatment-induced psychiatric symptoms have been identified as of yet[47]. While the presence of depression at baseline can be predictive of increased psychiatric burden and early treatment discontinuation, no apparent impact on the achievement of SVR in patients completing treatment has been reported[48,49]. Failure to clear HCV-RNA has however been associated with IFN-induced depression[44,50], and in these cases, serotonin-

norepinephrine reuptake inhibitors, bupropion and modafinil, may improve affective and neurovegetative symptoms of depression[51]. The efficacy of pre-emptive antidepressant therapy has been questioned in some studies[52], with the exception of patients with pre-existing subclinical depressive mood, mild cognitive disturbances, sadness, and reduced sleep[53].

In addition to depression, HCV is associated with social marginalization, impairment of intimate and family relationships, reduced sense of well-being, anger, inappropriate coping strategies, and stigma[51]. The pathogenesis of HCV-related neuropsychiatric symptoms is poorly understood and it still remains to be determined whether the occurrence of depression is a risk factor or a consequence of the infection.

While psychological and social factors are of primary importance, the evidence that depression is less frequently observed in HBV-infected patients treated with IFN compared to those with HCV infection suggests a relevant role of the virus itself in the development of neuropsychiatric symptoms[54].

FATIGUE

Central or cognitive fatigue is a subjective lack of energy remaining after rest, which is perceived as a sensation of physical and mental exhaustion, and is accompanied by a lack of motivation and difficulty in initiating and completing wilful actions. Severe fatigue is frequently associated with impaired cognitive function, with defects in concentration, attention tasks, and memory on neuropsychological testing. Irrespective of the severity of liver disease or viral replication rate, between 53% and 80% of HCV-infected patients complain of fatigue, often in conjunction with joint pain, restless leg syndrome, mood alterations, and headache[55]. Intriguingly, fatigue is worse in HCV-infected patients than in patients with comparable liver dysfunction of other aetiologies[56,57], and improves after antiviral treatment in the majority of patients. However, persistence of fatigue is observed in roughly one third of patients despite the decrease in viral load[58].

The association between HCV infection and fatigue has been questioned in earlier studies comparing HCV-infected patients with healthy blood donors[59], and also in studies reporting the lack of a prevalence of HCV infection among patients with chronic fatigue syndrome[60,61]. However, the latter condition, which differs from chronic fatigue, is a clinically defined complex disorder coupling disabling fatigue with neuropsychiatric symptoms, sore throat, muscle or joint pain, cervical or axillary lymphadenopathy, sleep disturbance, headache, and post-exertional exacerbation of malaise. In a cross-sectional observational study, 130 HCV-infected patients, 34% with cirrhosis, and 61 healthy controls completed a questionnaire to assess health-related quality of life (HRQL); 95 HCV patients also completed a fatigue questionnaire[62]. Chronic fatigue (defined as fatigue persisting for $\geqslant$ 6 mo) and chronic fatigue syndrome occurred in 71% and 27%, respectively, of HCV patients compared to 25% and 11% in the control population. Nevertheless, while more HCV patients reported chronic fatigue, the severity of fatigue experienced was similar between the two populations.

Establishing the causes of fatigue in patients with chronic HCV infection is difficult, although comorbid neuropsychiatric symptoms, liver fibrosis, and HCV neuroinvasion have been considered in several studies. However, while severe fatigue is generally an indication for antiviral treatment, "iatrogenic fatigue" is commonly encountered during interferon therapy, often leading to premature interruption of the treatment. A recent study addressed the effect of pegylated-INF/ribavirin treatment on fatigue in a cohort of patients with chronic HCV infection. At 24 wk after completion of treatment, the proportion of patients experiencing fatigue shifted from a baseline value of 53% to 33% in the group achieving SVR, with a median decrease at the visual analogue scale from 27 mm to 13 mm. Conversely, a non-significant decrease for the presence and the severity of fatigue was observed among non-responders[63].

SLEEP DISORDERS

Sleep has been defined as a behaviour which can be measured according to five dimensions, including duration, efficiency, timing, alertness, and satisfaction[64]. The assessment of the aforementioned dimensions represents a valuable indicator of sleep health. Sleep disturbance has a negative impact on innate immunity, impairs the response to infectious agent and vaccines, and is associated with increased susceptibility to acute infections and severity of symptoms[65]. In addition, the reported higher prevalence of altered sleep patterns in patients with acute and chronic infections, often occurring in conjunction with pain and fatigue in the setting of the so-called "sickness behaviour", reinforces the notion that the talk between the immune system and the brain is of great importance in regulating sleep architecture and other aspects of this behaviour.

Sleep disturbance with disordered circadian rhythm and reversal of day and night cycle has been frequently reported in patients with overt HE. In addition, abnormal sleep patterns have been detected in 50% of cirrhotic patients with minimal HE, as well as in those with non-cirrhotic chronic liver disease. To date, little attention has been devoted to sleep disturbance in chronic HCV-infected patients in spite of the frequent occurrence of complaints in sleep satisfaction/quality, a subjective judgement of sleep as good or poor. In a recent study, self-report sleep scales assessing quality, latency, duration, efficiency, and daytime dysfunction, in addition to sleep diaries and wrist actigraphy (as a measure of nocturnal activity and sleep-wake patterns), were combined to

examine a cohort of non-cirrhotic HCV patients with mild hepatic disease[66]. Not surprisingly, HCV-infected patients were dissatisfied with sleep quality, and exhibited altered sleep patterns, increased nocturnal activity, and poorer sleep efficiency, regardless of their individual viremic state. However, no alterations in the 24-h physical activity level were observed, and no correlation was found between fatigue and nocturnal activity. These findings are in contrast to the sleep disturbance observed in cirrhotic patients, which is characterized by fragmentation of sleep and reduced 24-h activity[67]. In addition, for delayed sleep phase syndrome, pronounced daytime sleepiness and frequent awakenings were observed in patients with minimal HE[68]. Taken together, among extra-hepatic manifestations of chronic HCV infection, sleep disruption entails a significant burden, and negatively impacts mood alterations[69], quality of life, and possibly fatigue. Further research is needed to clarify the pathophysiological mechanisms responsible for alterations in the circadian clock.

QUALITY OF LIFE

Quality of life can be defined as overall satisfaction with life, referring to wellbeing within the functional domain assessment of physical and mental health and social functioning. Chronic medical conditions are typically associated with a reduced quality of life as reported by patients, although measurement tools of such aspects have obvious limitations. Existing self-reporting questionnaires generate different scores according to different categories, such as general and mental health, social and physical functioning, pain, vitality, and impact of the disease[70]. There are no quality of life assessment scales specifically developed for HCV-infected subjects. SF-36 or its short version (SF-12) are typically used, since these methods have largely demonstrated high accuracy in detecting quality of life impairment in a wide range of chronic diseases. More than half of patients with chronic HCV infection complain of fatigue, lassitude, impaired concentration and memory, which are known to interfere negatively with quality of life at least as much as physical symptoms. Patients also report a reduced quality of life, which is frequently independent of the severity of liver involvement or virus replication rate. Fatigue, cognitive dysfunction and mood alterations have a profound effect on social and physical functioning, thus further impacting HRQL[71]. Lower quality of life scores have been described in HCV subjects compared to HBV-infected patients and healthy controls[56]. Interestingly, these findings seem not to be exclusively related to the psychological effect of HCV positive status awareness, since patients who are unaware of the infection perform better than patients who know their HCV status but worse than healthy individuals[72]. More recent studies that assessed HRQL using the SF-12 questionnaire in German HCV/HBV positive patients and healthy controls, confirmed a predominant impairment on both mental and physical quality of life in HCV subjects despite a more evident liver disease in HBV ones[73-75]. Whether these data reflect direct viral damage or are a consequence of chronic inflammation is still unclear, but viral infection itself is an important factor contributing to reduced HRQL.

SVR has been associated with an improvement in HRQL scores that can be due to both viral status and patient perception. Using the SF-36 scale, a comparison between HCV infected patients and healthy subjects revealed a lower HRQL at baseline, but a marked improvement was noted in patients responsive to IFN/antiviral treatment[76]. However, IFN treatment may also be associated with reduced quality of life, in particular during the initial phase of therapy, which is likely to be due to potential side effects of IFN injections both at physical and emotional levels[77].

THERAPY OF CHRONIC HCV INFECTION: PRESENT AND FUTURE

The combination of IFN and ribavirin no longer represents the therapeutic standard of care, at least for difficult-to-cure HCV-infected patients of gt-1 and gt-4[78]. The introduction of telaprevir and boceprevir has resulted in a higher rate of SVR, but these antiviral agents are being rapidly replaced by the new interferon-free, all-oral regimens that include direct-acting antivirals such as sofosbuvir, daclatasvir, and simeprevir, with or without ribavirin. In spite of the high costs that currently prevent their accessibility to all patients who may benefit from treatment, several of these regimens are under intensive clinical investigation and have been able to induce SVR in 90% or more of the patients, even in those with HCV re-infection and accelerated progression following liver transplant[79].

A careful assessment of the most appropriate therapeutic regimen for each specific clinical situation will require further multicentre trials. Additional points that need to be addressed include the identification of novel predictive factors, the adoption of new criteria to define the length of therapy, and the elucidation of resistance mechanisms that may result in the emergence of drug-resistant viral variants. Finally, the most eagerly awaited goal is the development of a safe and effective vaccine.

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TOPIC HIGHLIGHT

2015 Advances in Hepatitis C virus

Progress in the development of vaccines for hepatitis C virus infection

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Abstract

The hepatitis C virus (HCV), first described in 1989, is now a leading cause of liver cirrhosis and hepatocellular carcinoma. With more than 170 million people infected globally, this virus is a major public health issue. The current standard therapy is based on interferon in combination with ribavirin. This costly therapy often fails to completely clear the infection and is associated with adverse side effects. Recent anti-HCV therapies are interferon-free direct-acting antiviral (DAA) regimens for HCV, including simeprevir, sofosbuvir, and ledipasvir, which have effects on non-structural proteins. DAA regimens have several advantages, such as specifically targeting HCV viral replication, accompanied by very high sustained virological response rates and lower side effects like flu-like syndrome. These facts plus the fact that most HCV cases progress to chronic infection suggest the potential need for an efficient HCV vaccine. Different innovative methods, including methods based on peptide, recombinant protein, DNA, vector-based, and virus-like particles, have been introduced for the development of HCV vaccines. An extensive number of studies have been published on these vaccines, and some vaccines were even tested in clinical trials. In the current review, progress in the development of preventive and therapeutic vaccines against the HCV is reviewed in the context of peptide vaccines, recombinant protein vaccines, HCV-like particle, DNA vaccines and viral vectors expressing HCV genes.

Key words: Preventive vaccine; Therapeutic vaccine; Hepatitis C virus; Hepatitis C virus infection

Core tip: Chronic hepatitis C virus (HCV) infection occurs in about 75%-90% of acutely infected individuals. It may progress to liver cirrhosis or hepatocellular carcinoma. Despite satisfactory progress in the

management and treatment of chronic hepatitis C, it remains one of the most prominent viral infections worldwide. Although no reliable vaccine for it has yet been developed, researchers are trying to design and develop different types of vaccines to prevent HCV infection or to cure the chronic form of the disease. The current article provides an overview of the latest progress in the development of preventive and therapeutic vaccines against HCV infection in the context of peptide vaccines, recombinant protein vaccines, HCV-like particle, DNA vaccines, and viral vectors expressing HCV genes.

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INTRODUCTION

The hepatitis C virus (HCV) is an enveloped, positive-sensed, single-stranded RNA virus belonging to the *hepacivirus* genus of the *Flaviviridae* family[1]. Approximately 75% of acute HCV cases develop into chronic HCV infection, of which 3%-11% develop into liver cirrhosis within 20 years. This may eventually lead to liver failure or hepatocellular carcinoma (HCC) and may necessitate a liver transplant[2-4].

HCV is mainly transmitted through blood as in transfusion, injection drug use, organ transplantation, hemodialysis, or accidental exposure; however, unprotected sexual contact and vertical mother-to-child transmissions have also been documented[5,6].

First discovered in 1989[7], it is estimated that 3% of the world's population is infected with HCV. Infection rates vary from less than 1% to over 10% in different countries[8]. HCV antibody (anti-HCV) testing is used to assess the prevalence of HCV. Studies have reported that countries located in Africa and Asia have the highest anti-HCV rates, whereas industrialized countries, such as those located in North America, Western Europe, and Australia, have lower rates[9-11]. Although the incidence rate of HCV is decreasing in developed countries, mortality due to secondary liver disease from HCV infection is expected to continue to rise over the next 20 years[12]. HCV infection is more common in adult populations (people older than 15 years)[13]. With efficient screening strategies, better treatments, and the use of preventive vaccines, it is estimated that HCV could be eliminated in the next 15 to 20 years[13,14].

HCV strains are categorized into seven genotypes (1-7) based on their phylogenetic similarities and genome sequence. HCV genotype 1 is the most common, accounting for 46.2% of all cases worldwide, followed by genotype 3, which is responsible for 30.1% of all cases[15].

The majority of newly-infected people are asymptomatic or have a mild illness. Therefore, they are normally not aware of their condition[4]. Symptoms are anorexia, nausea, vomiting, abdominal discomfort, and jaundice. HCV rarely causes fulminant hepatic failure[16].

Drug therapy for HCV consists of the administration of interferon alpha (INF-α) and ribavirin and is associated with adverse side effects. A new class of drugs called direct-acting antivirals (DAA) is beginning to be used in combination with INF-α and ribavirin, resulting in an increase in their effectiveness. However, the drawbacks of DAAs are their high cost and more adverse side effects, such as fever, fatigue, chills, and depression[15]. Based on these factors and the fact that only a small percentage of HCV patients can be totally cured[17], the need for an effective HCV vaccine is apparent. Here, we review the obstacles and progress in the development of effective vaccines against HCV.

HCV VIROLOGY

HCV has an icosahedral capsid that consists of a single-stranded, positive-sensed RNA and is enveloped with E1 and E2 glycoproteins that are highly variable[18]. Scientists have been unable to culture HCV *in vitro*. This, in turn, makes studying its structure and replication processes difficult, but some progress has been made[19,20]. Virus replication occurs mainly in hepatocytes. It is estimated that each infected cell produces 50 virions daily. The virus also replicates in peripheral blood mononuclear cells (PBMCs) and may be responsible for high levels of immunologic disorders in chronic hepatitis C patients[1].

The genome of HCV contains a long open reading frame (ORF) encoding a 3000 amino acid length polyprotein that is cleaved into structural and non-structural (NS) proteins. A 5′ untranslated region functions as an internal ribosome entry site and permits direct binding of ribosomes to the start codon of the ORF, thus directing translation of ORF[21,22]. The first about 40 nucleotides of the RNA genome may be involved in RNA replication[23]. The 3′ untranslated region is comprised of four elements: (1) a short variable sequence; (2) a poly(U) tract of heterogeneous length; (3) a polypyrimidine stretch mainly containing U and a few Cs; and (4) a highly conserved 98 nucleotide sequence[24,25]. The poly(U) tract and the highly conserved region of 3′ UTR are essential for the infectivity of HCV[26].

Viral structural proteins include core protein (22 kDa), E1 (33 kDa), and E2 (70 kDa) glycoproteins, and the NS proteins NS2, NS3, NS4A, NS5A, and NS5B[27,28]. HCV core is a 22 kD basic protein responsible for forming the capsid structure. It has been implicated in HCV pathogenesis, the development of chronic infection, and immune response modulation. Core is the most conserved protein among various HCV genotypes,

and humoral responses against it are the first host responses to develop in an infected patient[29-31]. E1 and E2 play roles in cell entry. E2 glycoprotein interacts with the HCV receptor CD81 in liver cells. Moreover, low density lipoprotein (LDL) receptors, scavenger-receptor class B type 1 (SR-B1), and Claudin-1 act in the process of cell entry[18]. E2 contains three hypervariable regions (HVR) that play roles in cell entry, antibody binding, and outcome of the disease. In the HCV genome, P7 gene encodes the first NS protein and is located after the genes that encode structural proteins. It forms the ion channels that seem to be essential for the efficient assembly, release, and production of HCV[32].

P7 was the first NS protein located after structural proteins. Its function is unknown, but it forms the ion channels that seem to be essential for HCV production[19,29]. NS2 (23 kDa), which is located early after P7, is a membrane-associated protease that can cleave the NS2-3 junction. P7 has been shown to cooperate with other NS proteins and may facilitate viral assembly[29,33]. NS3 (67 kDa) is a serine protease with RNA helicase and nucleoside triphosphatase (NTPase) activities that cleaves HCV polyprotein at various junctions between NS proteins[29,33]. NS3 also regulates NS2 protease activity and inhibits type 1 interferon (IFN) production by blocking Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene 1 (RIG-I) signaling pathways[34,35]. NS4 is further cleaved into NS4A and NS4B. NS4A plays a role in the assembly of the HCV replication complex, viral pathogenesis, modulation of NS3 helicase, and facilitation of NS3 serine protease activities[29,33]. NS4B is responsible for the formation of cellular membranous webs that are the site of genomic replication in HCV. Any allelic variation in its sequence may strongly affect HCV replication[36].

The NS5A protein has RNA-binding activity and directly binds to NS5B, modulating its RNA polymerase activity[29,33,37]. The NS5B protein is an RNA-dependent RNA polymerase that lacks proofreading activity, thus giving rise to a high rate of replication errors and generating significant genetic diversity. This high rate of genetic variability produces a mixture of variants called quasispecies, even in a single infected individual[38].

NATURAL IMMUNE RESPONSES TO HCV

Figure 1 summarizes immune responses against HCV with a focus on both innate and adaptive immune responses. More details about the components and effects of such responses are found in the sections below.

Innate immunity and HCV

Innate immunity, the first system to respond to HCV infection, contains proinflammatory cytokines and a cellular component. In addition to resisting infection, innate immunity is also involved in provoking adaptive immunity[39]. Three arms of innate immunity that identify HCV infection as a threat are: (1) RIG-I-like receptors (RLRs); (2) TLRs; and (3) nucleotide oligomerization domain (NOD)-like receptors (NLRs)[40]. After HCV recognition, these arms send various downstream signals to induce the production of various cytokines, such as interleukins (IL) and IFNs, which create an antiviral state for uninfected cells, decrease HCV replication in infected cells, and link innate immunity to adaptive immunity[39]. The RIG-I pathway is activated shortly after infection. HCV promotes a structural change in RIG-I and leads to the activation of the transcription factors IFN regulatory factor 3 (IRF3) and nuclear factor kappa B (NFκB), which results in the production of type Ⅰ and Ⅲ IFNs[41].

TLRs can sense both viral nucleic acid and protein. TLR3 and 7 have been shown to play roles in intracellular HCV recognition[42]. TLR3 is found in hepatocytes and Kupffer cells and inhibits HCV replication[43,44]. TLR7 resides in plasmacytoid dendritic cells (pDCs) and Kupffer cells, leading to the production of type Ⅰ and Ⅲ IFNs and IL-1β and IL-18[45,46].

The NLR pathway results in the activation of inflammasome, a protein complex made of a sensor (NLR protein 3), the adaptor (ASC), and caspase 1 (a cellular protease)[47]. NLRs trigger the secretion of IL-1β and IL-18, which cause hepatic stellate cells to form fibrosis[48].

IFNs-α and β (type I IFNs) bind to similar receptors and activate the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, which in turn induces interferon-stimulated genes (ISGs). This pathway amplifies total IFN responses and degrades viral RNA[49]. Type Ⅲ IFNs include IFN-λ 1, 2, and 3 (IL-29 and IL-28 A and B, respectively). They activate the JAK/STAT pathway and induce ISGs, the same as type I IFNs; however, they bind to different receptors with distinct cellular distribution[50].

The role of IL-18 is still unclear in HCV infection, but it may suppress virus replication and induce the maturation of monocyte-derived dendritic cells (DCs)[51]. pDCs may be the major source of IFN-α production. Myeloid DCs (mDCs) migrate to lymphoid tissue to activate native T cells and bridge innate and adaptive immune responses[45].

Natural killer (NK) and NKT cells also play roles in HCV infection through IFN type Ⅱ (IFN-γ) production and cytotoxic destruction of infected cells[52,53]. IFN-γ activates Kupffer cells, and they release proinflammatory molecules, such as tumor necrosis factor (TNF)-α, galectin-9, and IL-18[51,54].

Humoral immunity and HCV

Humoral responses to HCV infection include B cell activation and production of antibodies that are primarily low titers of IgG1 and appear late in the course of the disease[55,56]. Antibodies can be detected

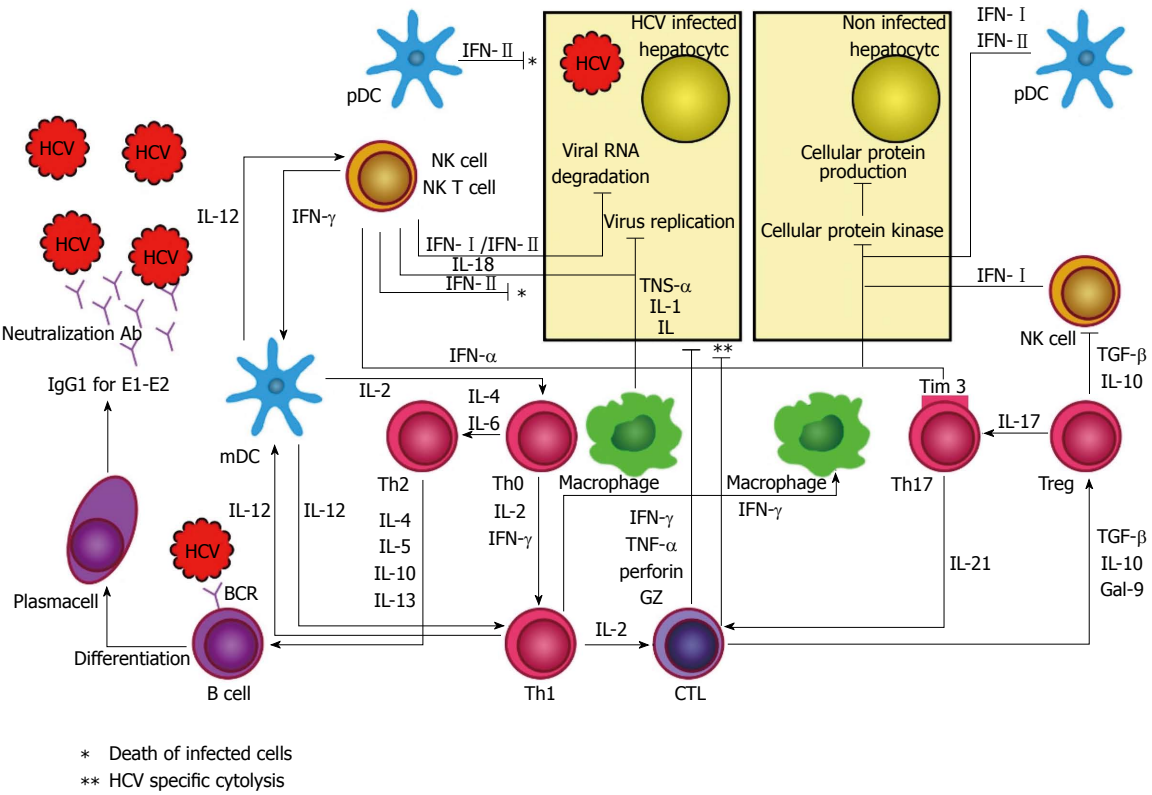

Figure 1 Summary of immune responses against hepatitis C virus. In innate immune response, NK cells are activated, which leads to the production of IFNs 1-11. This would inhibit the production of viral particles and destroy their genomes. In addition, in normal cells, antiviral effects are mediated through inhibition of protein synthesis. Adaptive immune responses work through the activation of T cells and production of cytokines that result in inhibition of the growth of the virus and elimination of the infected cells. B cells perform their actions through the production of neutralizing antibodies against the virus. Ab: Antibody; BCR: B cell receptor; CTL: cytotoxic T lymphocyte; E1: HCV E1 glycoprotein; E2: HCV E2 glycoprotein; GZ: Granzyme; HCV: Hepatitis C virus; IL: Interleukin; IFN: Interferon; mDC: Myeloid dendritic cell; NK cell: Natural killer cell; pDC: Plasmoid dendritic cell; Th: T helper cell; TGF: Transforming growth factor; TNF: Tumor necrosis factor.

after 7-10 wk of infection; however, viral RNA can be detected 1-3 wk after infection[57].

Although a few antibodies for E1 glycoprotein as well as NS proteins have been found, the main target for antibodies are the surface E2 glycoprotein[56,58,59]. Antibodies against NS proteins were found earlier in the disease course and with a greater magnitude. Nevertheless, E2 glycoprotein is still considered the main target for antibodies.

Recent studies have suggested that patients infected with HCV show an elevated level of activated B cells, and people with defects in antibodies experience rapid disease progression, emphasizing the role of humoral immunity in HCV[60-62]. Patients with hypogammaglobulinemia could spontaneously resolve acute hepatitis C, suggesting that humoral immunity is not essential for HCV clearance[63]. The activity of humoral immunity may contribute to some of the extra hepatic disorders associated with HCV, such as mixed cryoglobulimia and B-cell non-Hodgkin's lymphoma, although the mechanisms of these associations are still not well understood[64,65].

Cell mediated immunity and HCV

Cell immunity for HCV is composed of two arms: CD4+ and CD8+ T cells that are detectable in peripheral blood or the liver several weeks post-infection[66]. These T cells directly kill infected cells or produce soluble factors that clear the virus in a non-cytolytic manner. These cells may also directly damage the liver or attract non-specific inflammatory cells to accelerate liver inflammation[67].

Upon recognition of HCV through the major histocompatibility complex (MHC) class Ⅱ[68], CD4+ T cells (T helper) mainly produce IFN-γ that correlates with decreased HCV titer and transaminase levels[66]. These cells also produce IL-2 that helps the production of CD8+ (cytotoxic) T cells. Moreover, they can enhance B cell production. T helper actions are essential for spontaneous recovery during acute HCV[69]. It has been shown that T helper responses are not observed in patients developing chronic disease[70].

CD8+ T cells recognize HCV-infected cells through MHC class Ⅰ and lead to lysis of infected cells[68]. The role of MHC class Ⅰ in HCV recognition indicates that spontaneous clearance of HCV is related to the presence of some MHC class Ⅰ molecules, including human leukocyte antigen (HLA)-B*57, HLA-B*27, HLA-A*11, HLA-A*03, and HLA-Cw*01, and to the absence of HLA-Cw*04[71]. The mechanism of

the influence of these alleles in the clearance of HCV is unknown; however, some studies suggest that these allelic variants produce binding proteins to immunogenic or functionally conserved T cell epitopes[72]. There is evidence for the binding of some proteins to inhibitory receptors of NK cells. MHC class Ⅱ genes, such as DQB1*0301 and HLA-DRB1*1101, have also been associated with HCV clearance. Patients who lack efficient cytotoxic T cell response develop persistent hepatitis C infection, but the magnitude of the cytotoxic T cell response is associated with the clinical outcome[73,74].

CHALLENGES TO DEVELOPING A HCV VACCINE

HCV genetic variability

Studies suggest that the HCV first appeared over 1000 years ago and evolved in different environments into seven distinct genotypes and more than 100 subtypes[15,75,76]. HCV mutates at a rate of nearly one nucleotide per replication cycle. This is due in part to the lack of proofreading activity of NS5B RNA-dependent polymerase that causes an error rate of 10^{-3} to 10^{-5} per nucleotide per replication cycle[19]. These frequent mutations, in combination with a short viral half-life and rapid turnover (10^{10} to 10^{12} virions per day), lead to a high genetic variability that results in the presence of distinct but closely related HCV variants (known as quasispecies) in one infected individual[38]. E1 and E2 glycoproteins show the highest variability among HCV proteins[75]. It is estimated that HCV is 10 times more variable than the human immunodeficiency virus (HIV), and this clearly poses a significant challenge for successful vaccine development[77].

Immune evasion and HCV persistence

HCV can escape the immune system by inhibiting NK cells and IFN-α production and by mutating to evade antibody and CD8+ T cell recognition. The mutations in CD8+ T cell epitopes have been carefully described in many chimpanzee[78,79] and human studies[80-82].

During HCV replication, NS3 and NS4A block the signaling pathways of TLR3 and RIG-I, and that ultimately inhibits the production of type Ⅰ IFN[83-85]. This may lead to a decrease in NK cell activity through a lack of IL-15 production[86,87]. Core protein interferes with TNF-α and lymphotoxin signaling[88], and E1 and E2 glycoproteins build a glycan shield and facilitate cell-to-cell spread[89]. The NS5A protein could stimulate IL-8, inhibit double-stranded RNA-activated protein kinase, and interfere with 2′,5′-oligoadenylate synthetase (2′,5′-OAS), thereby interrupting the signaling of type 1 interferon[90,91]. The overexpression of HCV core protein interacts with the STAT1 SH2 domain and leads to the inhibition of IFN signaling[92,93]. The upregulation of ubiquitin-specific peptidase 18 (USP18) *via* long-term IFN stimulation suppresses ISG15 activation and JAK/STAT signaling, which can lead to type Ⅰ IFN stimulation resistance[94,95]. NK and NKT cells are present in liver tissue and produce IFN-γ and other cytokines to prime cellular immune responses[96].

HCV escapes humoral immunity by several mechanisms: (1) HCV binds to very low density lipoprotein, which facilitates its uptake by hepatocytes[97]; (2) three glycans at the CD81 binding site of E2 glycoprotein decrease the immunogenicity of the virus[98]; (3) CD81 and Claudin-1 allow HCV to infect surrounding cells through cell-to-cell contact[99]; and (4) constant mutations in HCV can induce interfering antibodies[100].

Another obstacle in developing an efficient vaccine is the persistence of HCV, mainly because of (1) rapid HCV escape mutations[101]; (2) the secretion of immune regulatory cytokines[102]; (3) T cell exhaustion and depletion[103,104]; and (4) T regulatory cell induction[105]. A further obstacle is the lack of a suitable animal model to study HCV.

HCV polymerase works at a high rate and lacks proof-reading activity, which leads to a rapid viral escape from humoral and cellular immunity[106,107]. Mutations in MHC class Ⅰ restricted epitopes that are targeted by cytotoxic T cells may lead to persistence[108,109].

IL-10, an immune-regulatory cytokine, increases in chronic hepatitis C. It is produced by T cells as well as monocyte and NK cells. IL-10 not only suppresses IFN-α and IFN-γ production and T cell proliferation but also promotes pDC cell apoptosis[110], resulting in liver damage in patients with chronic HCV infection[104]. Tumor growth factor-β also plays a role in HCV persistence[102].

In T cell exhaustion, T cells are in a state of dysfunction. This phenomenon can develop during many chronic infections like HCV. These cells lose their ability to produce IL-2, which is essential for the production of T cells, and show sustained expression of inhibitory receptors. These changes result in a loss of cytotoxicity and the cytokine production of cellular immunity[111]. Programmed cell death protein-1 (PD-1) plays important roles in CD8+ T cell exhaustion, and blocking PD-1 restores CD8+ T cell responses[112-114]. T lymphocyte antigen 4 [cytotoxic t lymphocyte (CTL)A4] also contributes to T cell exhaustion. Inhibition of both PD-1 and CTLA4 improves the restoration of T cell responses[115]. HCV induces T cell deletion by upregulating the pro-apoptotic molecule Bcl-2-interacting mediator (Bim) and downregulating the induced myeloid leukemia cell differentiation protein (Mcl-1)[104].

Regulatory T cells control the balance between host damage and viral control. These cells can induce immune-tolerance when excessive immune response could be harmful for the host. They inhibit the maturation of antigen presenting cells and the activation of T cells. Thus, they control auto-immunity

and immune responses[116]. Although higher T regulatory cell frequency has been observed in chronic HCV compared to self-resolving infection[117-119], a study in chimpanzees showed that HCV infection increases the number of T regulatory cells and their suppressive actions irrespective of the disease outcome[120].

HCV attacks mitochondria in liver cells and induces mitochondrial dysfunction and oxidative stress[121-123], resulting in the induction of cell autophagy and mitophagy, selective elimination of mitochondria, and the induction of mechanisms to eliminate dysfunctional mitochondria[124]. Autophagy proteins act as proviral factors that initiate the translation of HCV RNA in newly-infected cells, and autophagosomes will become sanctuaries for HCV replication away from host immune surveillance, which paves the way for chronic infection and liver failure[125,126]. Kim *et al*[127] suggested a similar role for mitophagy in the HCV life cycle.

Lack of an appropriate HCV model

Other than humans, chimpanzees[128] and a non-rodent small mammal named *Tupaiabelangeri*[129], also known as the tree shrew, are naturally susceptible to HCV infection. In addition to the mentioned animal models, much effort has been made to introduce genetically-manipulated animals, preferably mice or rats, for the study of HCV pathogenesis and vaccine design[130-133]. The results of chimpanzee studies are highly variable and difficult to interpret because of the genetic variability between animals and small sample sizes. Approximately 30%-40% of chimpanzees develop chronic HCV infection, whereas up to 85% of infected humans develop chronic HCV[134]. The high cost of acquiring and maintaining the animals, their limited availability, and ethical considerations are major drawbacks to using chimpanzees as HCV animal models[133]. The infection rate and viremia of tree shrews is low and rarely sustained; however, they can develop chronic hepatitis in some cases[135,136]. The limited availability of tree shrews, their high housing costs, and the lack of tupaia specific reagents (used to assess HCV-host interactions) limit their use for the study of HCV[128].

HCV is a highly complex virus. The study of immune responses was long hampered by the lack of a cell culture system or readily accessible small animal model. HCV can replicate in lymphoid cells or PBMCs but only for limited periods of time and very low viral loads[137]. The invention of HCV pseudo-particles (HCVpp) expressing unmodified E1E2 glycoproteins has provided a way for more thorough studies of HCV-specific antibodies[138], although the structure and neutralization of HCVpp are significantly different from natural HCV[139].

The first successful tissue model of HCV infection was developed in 2005 using the HCV/JFH1 cell culture system[140]. The whole genome of the JFH1 virus was separated from a Japanese patient with fulminant hepatitis; it was then multiplied with PCR, cloned, and named JFH1. The JFH1 virus is of the 2a genotype and has a full-length HCV genome. This culturing system efficiently maintains the replication of HCV in human hepatoma cell line Huh7 and produces fair titers of cell-cultured derived HCV particles (HCVcc) with natural infectious properties. This platform was further broadened to include HCV subtype 1a strain H77-S, which is more commonly associated with HCV complications, such as HCC and cirrhosis, and more resistant to IFN therapy than HCV genotype 2[141].

PREVENTIVE AND THERAPEUTIC VACCINES

Any preventive (prophylactic) or curative (therapeutic) immunogen that inhibits or treats the chronic phase of HCV infection can be referred to as an effective HCV vaccine, since HCV persistence results in liver failure and the development of clinical complications[142]. Patients reinfected with HCV demonstrated a significantly increased spontaneous viral clearance rate and broader T-cell responses compared with those who had been affected with primary infections[143]. Differences in viral kinetics and rates of clearance between primary and secondary infections strongly support the significance of memory immune responses in the clearance of natural infection and support the development of therapeutic and prophylactic vaccines[143,144].

Most chronic HCV infections exhibit lower HCV-specific T-cell responses compared with humoral responses. Thus, efforts have focused more on enhancing the T cell rather than humoral responses. In addition to inducing strong and cross-reactive neutralizing antiviral antibodies (humoral immunity), a suitable HCV vaccine should provoke long-lasting cellular immune responses involving helper CD4+and CD8+ T cells (cellular immunity)[31,145]. Although current therapeutic vaccine trials have successfully induced HCV-specific immune responses and transiently reduced viral RNA in subsets of patients, they have not completely cleared HCV infections or consistently reduced viral titers[145-147]. Here, different vaccine development strategies and the clinical trials in which they were tested were reviewed (Table 1).

Peptide vaccines

Peptide vaccines induce HCV specific T cell responses by presenting vaccine peptide to the T-cell receptor *via* HLA molecules. Therefore, they are HLA-specific and target only a selected subset of epitope sequences within the HCV genome. The high rate of genetic variation in viruses within populations and geographic regions limits the universal usage of these vaccines. They often contain multiple epitopes in order to induce broader T cell responses. Unfortunately, some peptides may induce T regulatory cells or immune tolerance[148].

Table 1 Hepatitis C virus theraputic and preventive vaccines tested in primates and humans

Type of vaccine	Lead author	Year	Vaccine	Tested in	Adjuvant	Ref.
Recombinant protein	Leroux-Roels	2004	Recombinant E1 (T2S-918/InnoVac-C)	Human *n* = 20	Alum	[160]
	Choo	1994	Recombinant E1 or E2	Chimpanzee *n* = 7	MF59	[153]
	Frey	2010	Recombinant E1 or E2	Human *n* = 60	MF59	[161]
	Drane	2009	Recombinant Core	Human *n* = 30	ISCOMATRIX	[163]
	Verstrepen	2011	Recombinant E1 or E2	Chimpanzee *n* = 4	Alum	[162]
	Puig	2004	Recombinant E1 or E2	Chimpanzee *n* = 2	Oil/water	[155]
Peptide	Firbas	2006	7 HLA-A2 restricted peptides (IC41)	Human *n* = 128	Poly-L-arginine	[149]
	Firbas	2010	7 HLA-A2 restricted peptides (IC41)	Human *n* = 54	Poly-L-arginine	[150]
	Yutani	2007	Peptide vaccine targeting E1, E2, NS3 and NS5A	Human *n* = 12	-	[145]
	Klade	2008	Synthetic peptide vaccine (core, NS3, NS4) (IC41)	Human *n* = 60	Poly-L-arginine	[146]
DNA vaccine	Forns	2000	DNA vaccine expressing E2 protein	Chimpanzee *n* = 2	-	[227]
	Sallberg	2009	NS3/4A expressing plasmid (ChronVac-C)	Human *n* = 12	-	[179]
	Alvarez-Lajonchere	2009	Plasmid (CIGB-230) expressing core, E1, E2 plus recombinant core protein	Human *n* = 15	-	[175]
Virally vectored	Rollier	2007	DNA/MVA	Chimpanzee *n* = 4	-	[201]
	Folgori	2006	Ad6/Ad24 + electroporated DNA	Chimpanzee *n* = 5	-	[191]
	Fattori	2006	Ad6/Ad6/ChAd32	Rhesus macaque *n* = 3	-	[194]
	Barnes	2012	Ad6/ChAd3	Human *n* = 30	-	[193]
	Youn	2008	Recombinant vaccinia	Chimpanzee *n* = 4	-	[207]
	Habersetzer	2009	Modified vaccinia Ankara virus expressing HCV NS3-NS5B (TG4040)	Human *n* = 15	-	[203]
Others	Elmowalid	2007	Virus like particles	Chimpanzee *n* = 4	AS01B	[215]
	Batdelgar	2008	V-5 Immunitor–heat- inactivated HCV antigens from HCV infected donors (tablet administered orally)	Human *n* = 10	-	[219]

HCV: Hepatitis C virus; E1: HCV E1 glycoprotein; E2: HCV E2 glycoprotein.

Intercell (Intercell AG, Vienna, Austria) developed a peptide vaccine, IC41, which consists of five synthetic peptides derived from conserved regions of core, NS3, and NS4 proteins of HCV genotypes 1 and 2 with a poly-L-arginine adjuvant. IC41 was assessed in 128 HLA-A2+ healthy volunteers in a phase I, dose-escalation, placebo-controlled randomized trial where it was proven to be safe and well tolerated. IC41 vaccination induced few interferon-producing cells and dose-dependent T cell immune responses[149]. Klade *et al*[146] sought to determine whether IC41 is able to induce HCV-specific T-cell responses in chronic hepatitis C patients. In their study, 60 HLA-A2+ chronic HCV patients who failed to respond to or relapsed from conventional therapy were sorted randomly into five groups to receive six doses of IC41 in a double-blind phase Ⅱ study. T-cell proliferation was recorded in up to 67% of patients, and IFN-γ responses were observed in up to 42% of patients in the IC41 vaccine groups. Moreover, three patients had transient declines of HCV serum RNA. Another randomized trial included 54 healthy subjects receiving either subcutaneous or intradermal IC41 vaccinations weekly or bi-weekly. Results showed that IC41 induced significant immunological responses; adding imiquimod to the formulation did not enhance immunogenicity and was associated with a lower immune response. Furthermore, intradermal injections caused more noticeable reactions, especially erythema and edema[150]. In another clinical trial conducted by Klade *et al*[151], 50 patients received eight intradermal IC41-plus-imiquimod vaccinations bi-weekly, and 21 patients received 16 subcutaneous vaccinations without imiquimod weekly. The first group showed a statistically significant HCV viral load decline, whereas no effect on HCV viral load was observed in the second group. A team in Japan also worked on HCV peptide vaccines and published their findings in two papers[145]. The first study assessed the functionality of a "personalized" vaccine containing four CD8+ A24 peptides combined with Freund's adjuvant in 12 HCV patients (genotype 1b) who had previously failed in standard IFN-based therapy. After the first vaccination, the rest of the vaccinations were carried out with only those peptides that produced responses in each participant. Although most patients developed peptide-specific T cell responses after the seventh injection, the authors observed a dose-dependent decrease in serum alanine aminotransferase (ALT) and HCV RNA levels in only five and three patients, respectively[145]. In the second study, another peptide vaccine composed of HCV core region (C35-44) peptides with ISA51, an emulsified incomplete Freund's adjuvant, was shown to be safe and well tolerated in a phase Ⅰ trial on 25

HCV non-responder patients. Around 60% of patients showed an increase in peptide-specific T cytotoxic responses. Also, a more than 30% improvement in ALT and a > 1 log reduction in viral load were observed in seven and two patients, respectively[152].

A phase Ⅰ, dose-escalation, placebo-controlled randomized control trial was performed to assess a virosome-based peptide vaccine containing NS3 peptides in 30 healthy participants; however, no results have been released so far (ClinicalTrials.gov Identifier: NCT00445419).

Recombinant protein vaccines

Recombinant protein vaccines are developed by isolating the gene(s) encoding the corresponding protein and cloning it/them in bacteria, yeast, or mammalian cells. This approach is based on the theory that an efficient number of viral epitopes can induce enough immune responses to develop protective immunity, generally including antibodies and CD4+ T cell responses. Some recombinant proteins are, by themselves, sufficient for developing a strong immune response, whereas others require adjuvants. The advantages of recombinant protein vaccines are that they do not contain the pathogen or its genetic material and no organism culture is required.

In 1994, a recombinant heterodimeric E1E2 vaccine was tested on seven chimpanzees, and sterilizing immunity against a homologous HCV strain was shown in five (Table 1)[153]. This vaccine was further tested against persistent infection by homologous and heterologous HCV strains and offered protection in 10/12 and 8/9 chimpanzees, respectively[154]. Puig *et al*[155] vaccinated two chimpanzees, one naïve and one recovered from acute HCV infection, with recombinant HCV E1E2 glycoproteins. High antibody titers to E1E2 in addition to strong T cell proliferative responses were observed. After challenge with HCV, viremia was delayed in both vaccinated animals compared to the non-immunized animals. The naïve chimpanzee became persistently infected, despite an initial strong immune responses, and the other chimpanzee had a significantly shorter and milder viremia compared to the re-infection of the non-vaccinated animals. Many other studies evaluating the efficacy of recombinant HCV vaccines in animals, especially chimpanzees, achieved similar results, which paved the way for recombinant HCV vaccine trials in humans (Table 1)[156-159].

The first prophylactic HCV vaccine tested in humans was a C-terminally shortened recombinant E1 protein with aluminum hydroxide (alum) adjuvant named T2S-918/InnoVac-C. This vaccine provoked a higher antibody response against E1 in healthy volunteers than in patients with persistent HCV infection. Studies on this vaccine, however, were ceased in 2007 (Table 1)[160].

Another recombinant E1E2 heterodimer vaccine with MF59C adjuvant was given to 60 healthy subjects in three different doses on day 0 and weeks 4, 24, and 48. Neutralizing antibodies and T cell responses to E1/E2 were observed in all subjects. Moreover, the produced antibodies were shown to block CD81, a major entry molecule for HCV. Although the vaccine was safe and well tolerated, its usage was hampered by technical difficulties in E1E2 protein manufacturing (Table 1)[161].

In 2011, Verstrepen *et al*[162] vaccinated four chimpanzees with either genotype 1b E1 or E2 recombinant with alum adjuvant and observed antibody responses in all subjects. Only antibodies against E1, however, were shown to neutralize HCV pseudo-particles. Furthermore, only chimpanzees vaccinated with E1 were protected from persistent hepatitis when challenged with a 1b strain of HCV.

Drane *et al*[163] evaluated a recombinant HCV core protein vaccine with ISCOMATRIX™ adjuvant. After satisfying results were obtained in rhesus macaques, a phase Ⅰ, placebo-controlled, dose-escalation clinical trial was conducted on 30 healthy volunteers. All participants except one demonstrated antibodies against HCV core protein. Despite the induction of strong CD4+ and CD8+ T cell responses in monkeys, T cell responses were observed in only two subjects who received the highest dose of vaccine. T cell cytokines were detected in all but one of the participants in the highest dose group.

InnoVAC-C, developed by Innogenetics Company (Innogenetics NV, Ghent, Belgium), was the first HCV therapeutic vaccine candidate based on recombinant E1 protein in alum adjuvant. The vaccine was administered to 26 of 35 chronically infected HCV patients (genotype 1) at weeks 0, 4, 8, 12 and 24, and others received a placebo (alum only). Then, 34 subjects received open-label E1 vaccines at weeks 50, 53, 56, 59, 62 and 65. Twenty-four subjects underwent liver biopsies before E1 vaccination and 17 mo later. According to Ishak scores, 9/24 patients (38%) improved two points or more, whereas others remained stable or their condition was exacerbated. The increase in anti-E1 specific antibody levels correlated with the relative decrease in ALT level. A significant *de novo* E1 specific T cell response was observed in all but three subjects[164]. Finally, studies on this vaccine led to a larger, placebo-controlled trial where 122 chronically HCV infected patients were randomly chosen to receive four courses of six injections over 3 years. Vaccination induced anti-E1 humoral and cellular immune responses, but contrary to earlier findings, the histological progression of liver disease was not halted[165]. The Innogenetics company in Belgium eventually abandoned this HCV vaccine program in 2008, and no further work has been published[166].

Another therapeutic vaccine, GI-5005, is based on recombinant core and NS3 proteins of HCV produced in yeast cells (*Saccharomyces cervisiae*). Studies of

in vitro and *in vivo* models demonstrated the robust immunogenicity of GI5005. Moreover, GI-5005 was evaluated in a phase Ⅰb clinical trial and displayed efficacy in patients with chronic HCV infection[167]. GI5005 was evaluated in combination with the standard therapy [pegylated (PEG)-IFN/ribavirin] in more than 250 chronic HCV-1 patients in a phase Ⅱ, placebo-controlled trial. Triple therapy was well tolerated with no increase in withdrawals due to adverse effects. Moreover, improved early virological responses were observed in all treated naïve patients as well as an increase in sustained virological response rates in prior non-responder patients[168].

DNA vaccines

In 2001, the first DNA vaccine was licensed for use to protect horses from West Nile virus[169]. The injection of a plasmid encoding antigenic HCV protein(s) or peptide epitope(s) resulted in protein expression *in vivo,* leading to both humoral and cellular immune responses in rhesus macaques[170-172]. DNA vaccination against HCV primes periphery T cells, which subsequently enter the liver and help clear the infection. Unfortunately, the initial success with the DNA vaccination in mice did not continue in humans, possibly because as the size of the immunized host increases, the efficacy of DNA uptake and gene expression decreases[173,174]. DNA vaccine includes the nucleotides encoding an antigenic portion of the virus, such as the viral core region or envelope region. The DNA vaccine is taken up by the host cell, transcribed, and translated to yield proteins. These proteins are processed *via* the endogenous MHC class 1 pathway and promoted *via* cell-mediated immunity (CMI). The first DNA-based vaccine to reach clinical trial for HCV infection was CIGB-230, which contained a mixture of Core/E1/E2-expressing plasmid with HCV core protein. The phase Ⅰ trial of this vaccine resulted in neutralizing antibody and HCV core specific T cell responses in the majority of patients. Furthermore, nearly half of the vaccinated individuals developed an improvement in liver histology with a reduction in fibrosis, even though viremia persisted[175]. Another clinical trial for CIGB-230 on 15 chronic HCV patients who were non-responsive to IFN treatment indicated that six patients developed weak *de novo* neutralizing antibody responses, and only one patient had a drop of > 1 log10 in viral load[176].

Due to the heterogeneity of HCV subtypes, a DNA vaccine including the most conserved regions (NS3 and NS4a) was designed. Through extensive codon modification, the DNA was effectively expressed *in vivo* and prime T helper cell type 1 (Th1) and CD8+ CTL responses in transgenic mice models[177,178]. This vaccine, named ChronVac-C and generated by the Tripep Company of Sweden, was the second DNA-based HCV vaccine to reach a human trial, and it was given to 12 chronic HCV patients through intramuscular electroporation. Initial results suggested safety and immunogenicity of vaccine. Two out of three patients receiving the highest dose showed a decrease in serum HCV RNA. Also, three patients who were under standard IFN-based therapy had an accelerated viral load clearance. Therefore, a treatment combining ChronVac-c with the standard IFN-based therapy was proposed for chronic HCV patients[179]. This vaccine is now in phase Ⅱ clinical trials for HCV infection (ClinicalTrials.gov Identifier: NCT01335711).

The nonstructural protein 3 (NS3) of the HCV, an attractive candidate for use in HCV vaccination, plays an important role in the viral life cycle while simultaneously interfering with the host defense system. The serine protease of NS3 cleaves the mitochondrial antiviral signaling proteins, thereby blocking IFN-β production[180-183]. NTPase/RNA helicase activities of NS3 may interfere with cellular RNA helicase-mediated functions, such as DNA replication, RNA transcription, and other cellular phenomena[183,184]. To overcome this challenge, Ratnoglik *et al*[185] constructed a series of DNA vaccines that express NS3 of HCV but with mutations to the catalytic triad of the serine protease and the NTPase/RNA helicase domain to eliminate the problems mentioned above. Immunization of BALB/c mice with resultant DNA vaccine candidates induced T cell immune responses similar to those induced by the wild type NS3 or NS3/4A complex.

Viral vectors expressing HCV genes

The main idea for vector-based vaccines is the use of manipulated viruses to deliver foreign genetic material to mammalian cells. Viruses can enter cells and express desired genes within the host while becoming non-pathogenic and non-replicative by deleting specific genes. Compared to peptide vaccines, vector-based vaccines introduce a broader range of viral epitopes and are presented through the MHC-I pathway and induce broader CD4+ and CD8+ T cell responses[186]. Adenoviral (Ad) vectors are the most used viral vectors for T-cell priming in non-human primates (NHPs) and humans[187]. Studies of HIV vaccination on rodents and NHPs established the use of Ad vectors as the most efficient approach to prime cytotoxic T cell responses[188]. Ad vaccines can stably express large foreign genes, remain outside the host's chromosome, and become replication defective by deleting the E1 locus[186,189]. However, one major drawback to using Ad vectors is a pre-existing immunity to them that results in vector clearance prior to induction of the desired immune response. To overcome this problem, rare subtypes, non-human adenoviruses (which are harmless to humans), or adenoviruses with altered surface proteins are used[187].

An Ad6-based vaccine, encoding the NS3, NS4A, NS4B, NS5A, and inactivated NS5B, was capable of inducing strong and specific T-cell responses against NS antigens of HCV in mice and rhesus macaques[190]. Okairos, Inc. (Rome, Italy) further investigated

this vaccine by testing it on five chimpanzees. Four chimpanzees showed protective levels after being challenged with a heterologous HCV virus *via* the induction of cross-reactive cellular responses[191]. After the safety of the Okairos vaccine was confirmed in a phase Ⅰ study[192], another trial was directed, but no results have been published yet (ClinicalTrials.gov Identifier: NCT01070407).

Based on the strength of this preclinical data, Barnes *et al*[193] conducted a phase Ⅰ clinical trial to assess chimpanzee Ad3 (ChAd3) and Ad6 vectors in 36 healthy volunteers and found that both vaccines induced specific T cell responses against multiple HCV proteins. These responses were capable of recognizing heterologous HCV strains (genotypes 1A and 3A). However, the vaccine was not as effective as predicted from the results in rhesus macaques, possibly due to higher levels of cross-reactive antibodies against Ads in humans[193,194].

Another approach in developing vector-based vaccines is the use of modified vaccinia Ankara (MVA) viruses to encode HCV specific genes. They are a highly attenuated poxvirus strain used in several vaccine designs for various conditions, including colorectal cancer, tuberculosis (TB), HIV, and melanoma[195-198]. The MVA genome is incapable of integrating into the host genome, as its life cycle takes place entirely in the host cytoplasm. Its use is more efficient than Ad-based vectors due to minimal pre-existing anti-MVA immunity[199,200].

The Transgene Company vaccinated chimpanzees with a heterologous prime-boost regimen with DNA and MVA encoding core, E1, E2, and NS3 genes (Table 1)[201]. Immunization induced strong HCV specific CD4+ and CD8+ T cell responses. In addition, all animals receiving prime-boost vaccines achieved high HCV-specific antibody titers, whereas those receiving the DNA prime alone did not. When challenged with a heterologous strain of HCV, three out of four chimpanzees developed chronic infection[201].

An MVA-based vaccine encoding HCV NS3, NS4, and NS5B genes was able to induce strong long-lasting cross-reactive HCV specific helper and cytotoxic T cells in HLA-A2.1 and HLA-B7.2 transgenic mice. An additional dose of vaccine given after 6 mo boosted these responses, suggesting that the vaccine can induce effective memory T cells[202]. This vaccine, also called TG4040, was evaluated in a dose-escalation trial of 15 chronically infected HCV patients. Results showed that six patients had a decline in HCV viral load (0.5-1.4 log10) that was associated with a significant CD8+ T-cell response[203]. A phase Ⅱ trial using the TG4040 vaccine in combination with standard PEG-IFN and ribavirin therapy has been completed, but no results have been published yet (ClinicalTrials.gov Identifier: NCT01055821).

Vector-based vaccines with strategies using neither modified vaccinia Ankara viruses nor adenoviruses are also under development. A study in HLA-A2.1-transgenic mice used the canary pox virus as a viral vector of two plasmids coding the entire HCV genome to boost the primary immunization with HCV DNA vaccine. Two months after a canary pox virus boost, strong cellular immune responses to HCV proteins were observed[204]. Another study evaluated the canary pox virus as a viral vector to boost the primary immunization with DNA vaccine. The results were similar to those previously discussed and revealed the potential for the canary pox virus as a viral vector for HCV vaccination[205]. Youn *et al* transdermally immunized four chimpanzees with recombinant vaccinia viruses expressing HCV genes developed by Marion Perkus at Virogenetics Inc.[206]. Upon challenge with infective doses of homologous HCV, two control chimpanzees that received only the parental vaccinia virus, developed chronic HCV infections, whereas the immunized animals developed strong IFN-γ-producing T cell responses and moderate T cell proliferative responses[207].

HCV-like particles

HCV virus-like particles (VLP) are safe and easily manufactured vectors for gene delivery that closely resemble the mature HCV structure. Therefore, it is possible to induce neutralizing antibodies and T cell responses against many epitopes using a single VLP-based vaccine. Their usage has been licensed for other viral infections, such as the hepatitis B virus (HBV) and human papillomavirus (HPV)[208-210]. Baumert *et al*[211] generated HCV-like particles (HCV-LPs) in insect cells using a recombinant baculovirus containing the complementary DNA for HCV structural proteins. Structural and antigenic compositions of HCV-LPs were similar to the original HCV, and after testing in mice, it was capable of inducing immune responses against various epitopes of HCV[212]. Another study showed the superior immunogenicity of the HCV-LP-based vaccine compared with the DNA vaccine in transgenic HLA2.1 and BLAB/c mice[213]. This vaccination approach was also tested on a NHP model - baboons. All 12 animals developed broad and long-lasting HCV-specific humoral and cellular immune responses. Adjuvants only marginally enhanced the immunogenicity of the HCV-LP based vaccine[214]. Four chimpanzees were also immunized with HCV-LPs, and they all developed HCV-specific T cell and proliferative lymphocyte responses against core, E1, and E2 proteins. After encountering an infectious HCV genotype, one chimpanzee developed transient viremia and the other three exhibited higher levels of viremia, but their viral levels became unquantifiable after 10 wk. Four naïve chimpanzees were also challenged with the same HCV genotype, and three developed persistent infections[215]. No results of human clinical trials testing HCV-like particles have been published to date.

Other strategies

Gowans *et al*[216] developed a novel peptide delivery system using autologous monocyte-derived DCs. In a phase I dose-escalation clinical trial of HCV patients, DCs were loaded and activated *ex vivo* with HCV-specific HLA-A2 restricted T cell epitope peptides. All six patients who received the vaccine exhibited weak *de novo* HCV-specific CD8+ T cell responses. However, the T cell responses were not continuous, and no change was observed in HCV RNA, anti-HCV antibody, or circulating cytokine levels.

An oral immunization method was introduced using attenuated *Salmonella typhimurium* as a carrier for delivery of HCV DNA to the lymphoid tissue of the gastrointestinal tract. Vaccination of HLA-A2.1 transgenic mice induced T cell specific responses in 86% of mice that continued for a minimum of 10 mo[217]. Another oral delivery approach was developed using attenuated salmonella carrying a plasmid coding HCV core and E2 proteins. Vaccination of BLAB/c mice induced cellular immune responses and antibodies against HCV core and E2 proteins[218]. Batdelger *et al*[219] evaluated the efficacy of V-5 immunitor tablets, comprising heat-inactivated HCV antigens from HBV and HCV infected donors' blood in 10 chronically infected HCV patients. All of the analyzed patients receiving a daily dose of this therapeutic vaccine tablet showed decreased liver enzymes after 1 mo. Moreover, no adverse side effects were observed. However, larger scale and longer studies are required to further evaluate this method and its safety and efficacy.

Plant-based vaccines are another novel approach for developing an easily producible and inexpensive HCV vaccine. The tobacco mosaic virus (TMV) was engineered to encode a chimeric protein containing E2 hyper variable region 1 (HVR1) protein and C-terminal of the B subunit of cholera toxin (CTB). Plants infected with this genetically engineered TMV produced the HVR1 peptide fused to the CTB. HVR-1 specific antibodies acquired from HCV infected individuals reacted with plant-derived HVR1/CTB. Moreover, the intranasal immunization of mice with plant-derived HVR1/CTB chimeric protein induced both anti-CTB and anti-HVR1 serum antibodies[220]. Another attempt at plant-based vaccines used an engineered cucumber mosaic virus (CMV) expressing a HCV 27-amino acid synthetic peptide. Rabbits fed engineered CMV-infected lettuce plants exhibited an HCV-specific humoral immune response[221].

Chimeric HBV/HCV vaccines are based on the fact that the HBV surface antigen is a safe VLP-forming delivery system that can carry various antigens, including HCV envelope proteins[222]. In 1999, Wu *et al*[223] fused truncated HCV core protein (HCc) with the HBV virus and compared its immunogenicity to HCV core protein alone in the sera of HCV-infected patients. The fused antigens exhibited antigenicity to both HBV and HCV, whereas HCc alone resulted in antigenicity to HCV. They tested the chimeric protein in rabbits and mice as well and concluded that chimeric protein is more immunogenic than HCc alone. In another study, HBV core protein (HBc) was fused with HCc or HCV NS3. The purified HBc/HCc and HBc/HCV NS3 were tested in mice. Both induced high humoral and cellular responses to HBV core protein. HBc/HCc led to low antibody and T cell responses to HCV core protein, whereas HBc/HCV NS3 induced higher levels of antibodies and no T cell responses to the NS3 epitope of HCV[224]. Chiron Corp. also invented a chimeric HBV/HCV vaccine using HCV envelope proteins fused with HBsAg[225]. Another chimeric vaccine was developed by replacing the N-terminal transmembrane domain of HBV surface antigen with the transmembrane domain of HCV E1 or E2 glycoproteins from genotype 1a. The chimeric particles were produced in the stably transduced ovary cells of Chinese hamsters and were used to vaccinate New Zealand rabbits. Vaccination resulted in a strong specific humoral response against both HBV and HCV envelope proteins. Induced antibodies were also able to neutralize HCV pseudo particles derived from heterologous HCV 1a, 1b, 2a, and 3 strains. Furthermore, responses against HBV were equivalent to those induced by the conventional HBV vaccine[226,227]. No clinical trials in chimpanzees or humans have evaluated such chimeric vaccines thus far.

CONCLUSION

This review focused on the development of different types of vaccines against HCV. The arms of the host immune system that are involved in the eradication of the virus were reviewed as well.

Different methods, including peptide, recombinant protein, DNA, vector-based, and VLPs, are currently used to develop and produce HCV vaccines. Peptide vaccines are the leading cause of HCV specific T cell responses. One associated problem is the high rate of genetic variation in viruses within populations and geographic regions. Recombinant protein vaccines are produced by specific gene(s) encoding the immune dominant protein(s). Viral epitopes are the leading cause of immune responses to develop protective immunity, generally including antibodies and CD4+ T cell responses. Vector-based vaccines are manipulated viruses that deliver foreign genetic material to mammalian cells. Viruses can enter cells and express desired genes within the host while becoming non-pathogenic and non-replicative by deleting specific genes. HCV VLPs are safe and easily manufactured vectors for gene delivery that closely resemble the mature HCV structure.

The challenge for the development of an efficient vaccine with relatively low side effects is still yet to be overcome. Although significant steps have been taken in accomplishing this goal, no promising results

have been achieved to date. In part, this depends on the current understanding of the interaction of the virus and the host. Therefore, a more thorough understanding of the host immunity in the context of HCV infection is expected to lead to better results. Of course, other areas of biomedical sciences can greatly contribute to these studies. For instance, providing a suitable animal model for preliminary studies of the newly designed vaccines is crucial.

An effective vaccine against HCV may need to target another cellular process for HCV entry and release or may need to target another sequence of the virus genome. However, due to the high HCV mutation rate, that is not necessarily the best option. A balance between its efficiency in targeting a sequence that can inhibit further growth and a sequence that is highly conserved among different HCV genomes must be found. Alternatively, an efficient vaccine may target several cellular and molecular processes simultaneously. Vaccines may also be accompanied by different adjuvants to increase their efficiency rates. Moreover, other approaches may rely on using other targets, *e.g.*, targeting mRNAs by using synthetic interfering RNAs, such as microRNAs and small interfering RNAs.

In addition to attempts to develop vaccines, integrated programs should be carried out to study their effects in individuals; for instance, in which intervals of injection (in case of several infections), in which age group or sex, and with which genetic background, especially regarding HLA types and genotype variations in different positions of cytokine genes, is it more efficient.

Another seemingly important fact is the relative danger of exposure to HCV or its related particles during studies. This requires laboratories with high safety levels, which may not be readily available everywhere, and this can reduce the pace of studies on HCV. It is expected that ongoing studies will eventually lead to the development of more reliable vaccine types, and this, in turn will reduce worldwide incidence and prevalence rates of HCV infection.

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TOPIC HIGHLIGHT

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Diagnostic and therapeutic management of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is an increasing health problem, representing the second cause of cancer-related mortality worldwide. The major risk factor for HCC is cirrhosis. In developing countries, viral hepatitis represent the major risk factor, whereas in developed countries, the epidemic of obesity, diabetes and nonalcoholic steatohepatitis contribute to the observed increase in HCC incidence. Cirrhotic patients are recommended to undergo HCC surveillance by abdominal ultrasounds at 6-mo intervals. The current diagnostic algorithms for HCC rely on typical radiological hallmarks in dynamic contrast-enhanced imaging, while the use of α-fetoprotein as an independent tool for HCC surveillance is not recommended by current guidelines due to its low sensitivity and specificity. Early diagnosis is crucial for curative treatments. Surgical resection, radiofrequency ablation and liver transplantation are considered the cornerstones of curative therapy, while for patients with more advanced HCC recommended options include sorafenib and trans-arterial chemo-embolization. A multidisciplinary team, consisting of hepatologists, surgeons, radiologists, oncologists and pathologists, is fundamental for a correct management. In this paper, we review the diagnostic and therapeutic management of HCC, with a focus on the most recent evidences and recommendations from guidelines.

Key words: Cancer; Chronic hepatitis; Cirrhosis; Hepatitis B virus; Hepatocellular carcinoma; Hepatitis C virus; Liver

Core tip: Hepatocellular carcinoma is an increasing health problem, representing the second cause of cancer-related mortality worldwide. The major risk factor for hepatocellular carcinoma (HCC) is cirrhosis. Early diagnosis is crucial for curative treatments. As a consequence, patients at risk of developing HCC should undergo surveillance programs in order to detect HCC in the initial stage. Surgical resection, radiofrequency ablation and liver transplantation are considered the

cornerstones of curative therapy, while for patients with more advanced HCC recommended options include sorafenib and trans-arterial chemo-embolization.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide. More than 700000 new cases are diagnosed every year throughout the world and high incidence to mortality ratio (1.07) makes HCC the second most common cause of cancer-related deaths worldwide[1].

Although the majority of cases occur in Asia and Africa, the incidence has increased even in the developed world. The geographical variation in the incidence of HCC is mostly related with the different prevalence of major risk factors for HCC, such as hepatitis C virus (HCV) and hepatitis B virus (HBV) infection[2]. In developed countries, the epidemic of obesity, diabetes and nonalcoholic steatohepatitis (NASH) is also believed to contribute to the observed increase in HCC incidence[3]. However, the overriding risk factor for HCC, which is responsible for HCC in 80%-90% of cases regardless of etiology, is the presence of cirrhosis[4,5].

By recognizing the risk factors for HCC, high-risk groups can be identified and followed up with screening strategies. In fact, the management of high-risk patients with screening and surveillance has the real potential to detect HCC early and improve patient outcomes. When HCC is detected earlier, patients are candidates to receive curative treatments.

In this paper, we review the diagnostic and therapeutic management of HCC, with a focus on the most recent evidences and recommendations from guidelines.

DIAGNOSIS

Hepatic nodules can be detected on Ultrasounds (US), including contrast-enhanced US (CEUS), or on other noninvasive techniques, such as contrast-enhanced computerized tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET)-CT. The typical vascular profile of HCC on dynamic imaging is characterized by early arterial phase enhancement followed by loss of enhancement in the portal venous phase and delayed phase in comparison to the surrounding liver[6].

Moreover, molecular biomarkers could potentially be used for diagnosis, as well as prognostic evaluation and may help defining the individualized therapeutic approach to HCC. Figure 1 illustrates the diagnostic algorithms endorsed by the American Association for the Study of Liver Diseases (AASLD)[7].

US

Early diagnosis of HCC is important because, as expected, treatment is more effective when the tumor is small[8-10]. Dysplastic nodules (DNs) may develop into carcinoma[11]. Early detection of DNs with small areas of HCC is fundamental for effective treatment.

Cirrhotic patients are recommended to undergo HCC surveillance by abdominal US at 6-month intervals. However, the diagnosis of small HCC nodules may be challenging, as it is often difficult to differentiate benign from malignant lesions in the context of nodular cirrhosis; moreover, US depends on the operator and has limited sensitivity in obese patients. On the other hand, US is less expensive than other techniques and is radiation-free.

For HCC, a stepwise process of carcinogenesis has been proposed, involving a progression from regenerative nodules (RNs) to low-grade DNs or high-grade DNs to DNs with a focus of HCC and finally to HCC. This progression has been suggested to correlate with changes in the blood supply and perfusion of the nodules, which may be used to differentiate focal liver lesions[8,12-14]. Recently, SonoVue, a blood-pool marker used in CEUS, has been reported to help distinguishing RNs from small HCC based on the different enhancement pattern[15-18]. RN has an intranodular blood supply that is similar to the surrounding parenchyma. On the other hand, HCC usually exhibits an enhancement pattern in the arterial phase and washout in the late phase[19-26]. DN-HCC nodules have a mixed enhancement behavior, as they are composed of two different cells, high-grade HCC and atypical hepatic cells and their enhancement features are partially similar to HCC and partially similar to RNs[27]. Of interest, CEUS has been suggested to promote the diagnostic accuracy of biopsy, decreasing the false-negative rate for malignant lesions. In fact, CEUS may be used to identify the areas of viable tumor[27]. A biopsy of DN-HCC without CEUS guidance is more likely to give false-negative results, significantly affecting the possibility to early detect and treat HCC.

CT

CT is largely used in most centers to make the radiological diagnosis of HCC after a liver nodule is detected on US. Most centers use a four-phase multidetector CT (MDCT) scan, which consists of a non-enhanced phase, an arterial phase (which occurs 20-30 s after contrast injection), a portal venous phase (6580 s after contrast injection) and a delayed phase. On the four-phase CT, HCC classically appears as a hyper-attenuated lesion in the arterial phase, with loss

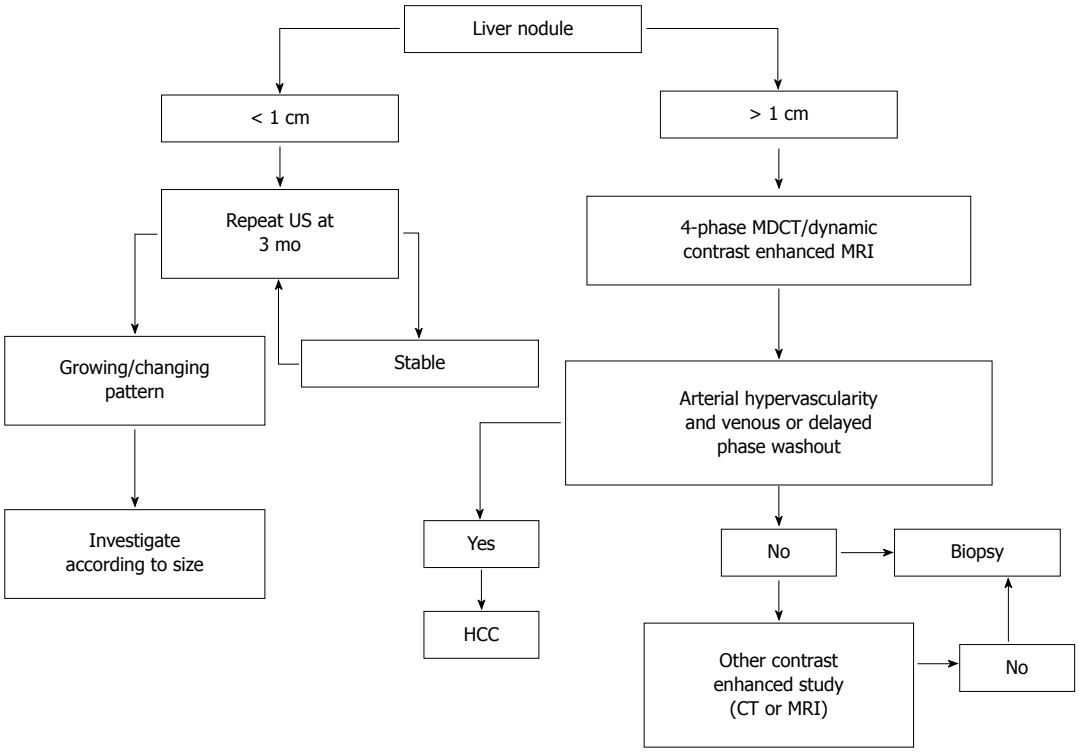

Figure 1 American Association for the Study of Liver Diseases diagnostic algorithm for suspected hepatocellular carcinoma (adapted from[7]). CT: Computed tomography; MDCT: Multidetector CT; MRI: Magnetic resonance imaging; US: Ultrasound; HCC: Hepatocellular carcinoma.

of enhancement termed rapid washout in the portal venous and/or delayed phase. CT has high specificity but variable sensitivity for detecting HCC. In a systematic review, traditional spiral CT was reported to have a specificity of 93% but a sensitivity of only 68% in diagnosing HCC. A more recent review assessing the diagnostic accuracy of the 64-slice MDCT technology *vs* spiral CT found improved sensitivity (65%-79% compared to 37%-54%), with similar specificity (above 90%)[28]. However, sensitivity dropped to 33.45% for nodules smaller than 1 cm.

MRI

MRI is an appealing imaging technique, since it does not use ionizing radiation. MRI allows the differentiation between tumoral and normal liver parenchyma using magnetic fields, even without a contrast media[29]. Traditional dynamic contrast-enhanced MRI of the liver is performed using gadolinium chelates. In gadolinium-enhanced MRI, the typical HCC lesion is hyper-intense on T1-weighted images during the arterial phase and exhibits rapid washout during portal venous and delayed phases[12,30,31]. The sensitivity of standard gadolinium-enhanced MRI is around 90%, with a specificity of at least 95% for the detection of HCC greater than 2 cm in diameter[32]. Dynamic MRI appears superior to CT for the detection of HCC nodules[33,34], but its sensitivity is highly affected by the lesion size, being as low as 30% in the case of lesions smaller than 2 cm[35,36].

Specific contrast agents have been developed to improve the sensitivity of MRI for HCC, including the "dual contrast" agents (gadolinium-ethoxybenzyl-diethylenetriaminepentaacetic acid and gadobenate dimeglumine), which work both as markers of hepato-biliary excretion and vascularization. HCC nodules imaged with these contrast agents do not exhibit uptake, unlike benign nodules on the delayed phase[37]. While it appears performing similarly to MDCT for lesions larger than 2 cm, enhanced MRI might be more sensitive for lesions smaller than 1 cm[38].

Nuclear imaging

Both ^{18}F-FDG and 11C-acetate PET imaging have been used for HCC detection and staging[39-41]. However, up to 40%-50% of HCC are not sensitive to ^{18}F-FDG PET, because of their high expression of the glucose-6-phosphatase enzyme, which prevents intracellular accumulation of ^{18}F-FDG[39]. On the other hand, 11C-acetate, which is believed to mainly participate in fatty acid synthesis in the liver, has been suggested to have increased sensitivity and specificity in comparison to ^{18}F-FDG[39,41,42]. However, several studies have reported that 11C-acetate PET does not properly differentiate HCC from benign lesions[41,43-45], because the latter also accumulate 11C-acetate. Some recent studies[41,44-47] have suggested dynamic PET with kinetic modeling to be a promising tool to differentiate benign hepatic tumors from HCC.

Biomarkers

A-fetoprotein (AFP) is the most widely used and

broadly known biomarker for HCC, but its use as an independent tool for HCC surveillance is not recommended by current guidelines due to its low sensitivity and specificity. In the past, a significant concentration of AFP in the serum of a patient with liver cirrhosis and a suspicious mass in the liver larger than 2 cm was sufficient to diagnose HCC[48]. However, the current diagnostic algorithms endorsed by the AASLD and the European Association for the Study of the Liver strictly rely on typical radiological hallmarks in dynamic contrast-enhanced imaging apart from biomarkers[7,48,49].

Three serum biomarkers have been suggested as tools to determine the risk of liver cancer in high-risk populations worldwide: AFP, the ratio of lecithin-bound AFP to total AFP (AFP-L3), and des-gamma-carboxyprothrombin (DCP). However, most studies on the performance of biomarkers in HCC detection have not been performed in a surveillance setting but compared levels of predefined biomarkers in patients with HCC with a control group, in most cases represented by patients with chronic liver diseases. A randomized controlled study performed in a high-risk population in China showed that screening by AFP measurement led to earlier diagnosis of HCC but had no impact on mortality[50]. On the other hand, semiannual screening for HCC by AFP measurement in a population-based study in Alaska was effective in detecting HCC at early stages and significantly prolonged survival rates[51].

A meta-analysis on the performance of AFP in diagnosing HCC included seven studies and revealed a pooled sensitivity of 66% with a specificity of 86% [area under curve (AUC) = 0.87][52]. In a further meta-analysis including ten studies the pooled sensitivity of AFP for the diagnosis of HCC was 51.9%, with a specificity of 94% (AUC = 0.81)[53]. A major drawback of AFP as a surveillance tool is that serum levels are influenced by the activity of the underlying liver disease and therefore increased in patients with elevated alanine aminotransferase (ALT) levels, even in the absence of HCC, as shown in the HALT-C trial[54]. Furthermore, HCC biology is quite heterogeneous, with only a proportion of patients with HCC having elevated AFP serum levels, leading to low sensitivity of the marker. As a consequence, new complementary markers have been studied. The clinical utility of high-sensitivity AFP-L3 (hs-AFP-L3) in early prediction of HCC development in patients with chronic HBV or HCV infection was recently evaluated in a large Japanese study. Even in subjects with low AFP levels and without suspicious ultrasound findings, an elevation of hs-AFP-L3 was an early predictor of HCC development: in fact, hs-AFP-L3 increased in 34.3% of patients one year prior to diagnosis of HCC[55,56]. Numerous studies have investigated the performance of other markers, including α-l-fucosidase[57], glypican-3 (GPC-3), insulin-like growth factor[58], vascular endothelial growth factor (VEGF), or Dickkopf-1[59], Golgi protein 73 (GP73), interleukin-6 (IL-6) and squamous cell carcinoma antigen (SCCA)[60]. In a study comparing 144 patients with HCC to 152 patients with cirrhosis and 56 healthy controls, GP73 had a sensitivity of 62% and a specificity of 88% at a cut-off of 10 relative units[61]. Another study, including 4217 subjects (789 with HCC), revealed a sensitivity of 74.6% and a specificity of 97.4% at a cut-off of 8.5 relative units[62]. Using different cut-off values, IL-6 sensitivity ranged from 46% to 73% with a specificity of 87% to 95%[60,63,64]; in a large study including 961 patients, SCCA had a sensitivity of 42% and specificity of 83% using a cut-off of 3.8 ng/mL[60,65].

Serum IL-17 levels have been reported to be elevated in HCC patients[66]. In a retrospective study, Liu *et al*[67] found that plasma IL-17 concentration had a sensitivity of 74.3% and specificity of 75.6% (AUC = 0.86) at the cut-off value of 4.23 ng/L; however, the diagnostic accuracy of IL-17 was lower than AFP, which had a sensitivity and specificity of 100% and 66% respectively, at the cut-off value of 10.25 mg/L (AUC = 0.96).

Osteopontin, an integrin-binding glycol-phosphoprotein, was investigated in seven studies summarized in a meta-analysis[52]. The pooled sensitivity of osteopontin for HCC was 86% with a specificity of 86%, showing a diagnostic accuracy similar to that of AFP; however, further validation studies are needed before recommending the use of this biomarker in clinical practice.

Some studies have investigated the combined diagnostic performance of the three more validated non-invasive biomarkers used in HCC, namely AFP, AFP-L3 and DCP. By comparing 164 European patients with HCC to 422 subjects with chronic liver disease, a significant increase in AFP serum levels was shown in those with advanced HCC and viral hepatitis, while DCP was more frequently elevated in those with early-stage and NASH-associated HCC. Neither of the two parameters, if taken alone, could independently identify more than 30% of patients with HCC but combination of AFP (cut-off 10 ng/mL) and DCP (cut-off 5 ng/mL) showed a sensitivity of 55% for early stage HCC and 78% for all stages[68]. A further increase in sensitivity (up to 84%) was observed by adding AFP-L3[69]. The additional use of clinical variables, like age and gender, further improved the performance of the model[70,71].

A number of signal transduction pathways have been recognized as critical players in the pathophysiology of hepatocarcinogenesis, including the Wnt/β-Catenin pathway, the p53 pathway, the tumor suppressor retinoblastoma protein pRb1 pathway, the mitogen-activated protein kinase pathway, the Ras pathway, JAK/STAT signaling, mechanisms of cellular stress response, like heat shock proteins, epidermal growth factor receptor and transforming growth factor-β signaling[72,73].

Gene expression profiling of peripheral blood mono-

nuclear cells using microarrays and bioinformatics-driven data analysis identified a blood-based signature of three genes, namely Chemokine (C-X-C motif) receptor 2 (CXCR2), C-C chemokine receptor type 2 (CCR2) and E1A Binding Protein P400 (EP400), able to predict HCC with a sensitivity of 93% and a specificity of 89%[74]. High-throughput metabolomics technology with the comprehensive analysis of small molecular metabolites may identify serum metabolic profiles to be used as biomarkers in HCC diagnosis. Molecular signatures may help to distinguish dysplastic nodules from well-differentiated HCC. In Asian and Western patients with HCV infection, specific gene signatures have been reported to accurately reflect the pathological progression of disease from cirrhosis to dysplasia to early and advanced HCC[75,76]. Moreover, a three-gene set including glypican3 (GPC3; 18-fold increase in HCC, $P = 0.01$), LYVE1 (12-fold decrease in HCC, $P = 0.0001$) and surviving (2.2-fold increase in HCC, $P = 0.02$) had an accuracy of 94% to distinguish DNs from early HCC in HCV-related cirrhosis[77]. Heat shock protein 70 and cyclase-associated protein 2 are other tissue biomarkers potentially useful to in the diagnosis of HCC[78,79].

As for novel biomarkers, microRNA (miRNA) have received particular attention[80]. miRNAs are small non-coding and evolutionary conserved RNA molecules that serve as posttranscriptional regulators of mRNA expression and interfere with mRNA translation to protein[81]. miRNAs are able to conserve their function into the cell by regulating the expression of a target population of molecules; moreover, they can be released from the cell both in combination with other proteins or as a free molecule[82-88].

Differences in miRNA expression patterns in several malignant conditions, including HCC, have been found[89-91]. In particular, three miRNAs, miR-122, miR-192 and miR-199a/b-3p, account for more than 70% of total miRNA released by normal liver tissue[91]. In HCC, a broad spectrum of changes in microRNAoma has been reported[91-94], suggesting that miRNAs may potentially become valid biomarkers in HCC. To improve the diagnostic utility of miRNAs in HCC, Li *et al*[95] performed deep sequencing in pooled samples from patients with chronic HBV patients, HCC and controls with and without cancer. They recognized a pattern of 6 miRNA differentially expressed in patients with HCC. The use of three miRNAs (miR-25, miR-375 and let7f) had a sensitivity of 97.9% and a specificity of 99.1% to discriminate between controls and HCC patients. Of interest, the use of two miRNAs (miR-10a and miR-125b) could adequately discriminate the cohort with chronic HBV and HBV-associated HCC with an AUC of 99.2% (sensitivity 98.5% and specificity 98.5%)[95]. In another study, a panel of 7 miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) provided a high diagnostic accuracy for the identification of HBV-related HCC[96], with a sensitivity of 81.8% and a specificity of 83.5%, independently of disease stage. However, the expression of selected miRNA was analyzed using RT-PCR, which may be critical for clinical translation of these findings[96]. miR-21, which is the most frequently deregulated miRNA in cancer, was found at higher level both in sera and plasma of HCC patients[97,98], while other studies showed no significant differences[99,100]. Similarly, miR-122, the most abundant miRNA in the liver, was also found at high level in sera of HCC patients[98,99]. Other inflammatory conditions of the liver, such as acute and chronic hepatitis and NASH may strongly influence miR-122 levels[101,102]. In this setting, further studies are required to establish the capability of these biomarkers to discriminate between chronic liver diseases and HCC.

STAGING

A number of staging systems have been used for HCC, even if the Barcelona Clinic Liver Cancer (BCLC) staging system is the most extensively used in clinical practice. BCLC staging system includes the evaluation of tumor stage, cirrhosis stage, functional performance status (PS) and it links staging with a treatment algorithm[103]. Moreover, the BCLC staging system was endorsed by both the American and European liver society and validated in European and American cohorts[104,105].

Early stage HCC (stage 0) has the best prognosis and is characterized by the presence of one lesion smaller than 2 cm in diameter, with no evidence of vascular invasion, in patients with stable cirrhosis (Child-Pugh class A).

Patients with stage A HCC could present with either a solitary lesion or up to three lesions of less than 3 cm in diameter. These patients have relatively preserved liver function (Child-Pugh class A or B) and good functional status (PS 0-2). The 5-year survival rate is 50%-75%; as reported in Figure 2, treatment may be different based on the presence of portal hypertension, the degree of liver dysfunction and other comorbidities.

Patients with intermediate stage HCC (stage B) have Child-Pugh class A or B cirrhosis, good functional status (PS 0) and multinodular HCC, with no evidence of vascular invasion. Patients with evidence of vascular invasion or extra-hepatic spread have advanced stage HCC (stage C). These patients typically have worse functional status (PS 1 or 2).

Patients with terminal stage HCC (stage D) present with decompensated cirrhosis (Child-Pugh class C), poor functional status (PS > 2), and advanced tumor growth (vascular invasion and/or extra-hepatic spread). Unfortunately, these patients receive no benefit from the currently available therapies, and survival is usually around 3 mo. Figure 2 illustrate the BCLC staging system and treatment strategies for HCC.

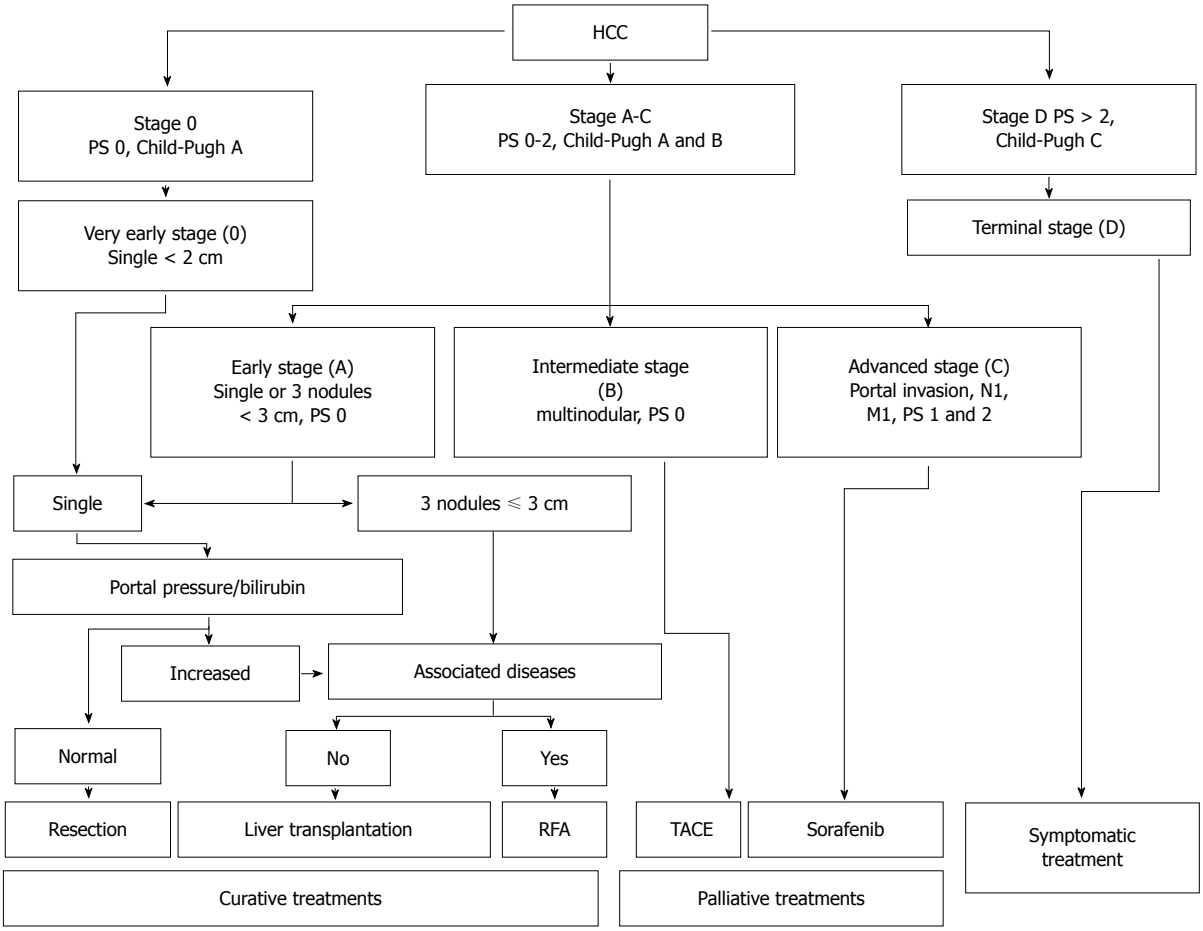

Figure 2 Barcelona Clinic Liver Cancer staging system and treatment strategy for hepatocellular carcinoma (adapted from Ref. [49]). M: Metastasis classification; N: Node classification; PS: Performance status; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization.

TREATMENT

Several therapeutic options are available for HCC, depending on HCC stage, liver function, comorbidities, and local clinical expertise. A multidisciplinary team, consisting of hepatologists, surgeons, radiologists, oncologists and pathologists, is fundamental for a correct management.

Surgery

The evaluation of liver functional reserve before hepatectomy is fundamental the maximum amount of liver mass that can be safely removed: on the one hand, liver functional overestimation may lead to hepatic failure; on the other hand, poor resection may significantly increase the risk of early recurrence of HCC.

The most important methods to assess liver function before surgery are the galactose tolerance test, ^{99}mTc-galactosyl human serum albumin liver scintigraphy and the indocyanine green (ICG) test. Makuuchi's selection criteria for hepatectomy rely on three factors, ascites, serum bilirubin and ICG retention rate at 15 min (ICGR15)[106]. Patients are considered eligible for liver resection if they have no ascites and if serum bilirubin is ⩽ 2 mg/dL. Patients with total bilirubin of 1.1-1.9 mg/dL can undergo partial liver resection; for patients with serum bilirubin ⩽ 1 mg/dL, the extent of resection is based on ICGR15: (1) resection of 2/3 of the total liver volume (TLV) (*e.g.*, right lobectomy) in patients with normal ICGR15 of < 10%; (2) resection of 1/3 of the TLV (*e.g.*, left lobectomy) in patients with ICGR15 of 10%-19%; (3) resection of 1/6 of the TLV in patients with ICGR15 of 20%-29%; and (4) limited resection or enucleation in patients whit ICGR15 ⩾ 30%.

A surgical mortality rate of 0% has been reported in 1056 consecutive hepatic resections performed in accordance with these criteria[107].

In patients with portal venous invasion[108], the area supplied by the portal vein branches should be systemically removed as much as possible within the acceptable range of liver function. In this contest, systematic subsegmentectomy has been developed to overcome the potential incompatibility between the attempt to remove cancer and the need to preserve liver function[109]. Tumor stage, tumor size, number of tumors and capsule formation predict recurrence-free survival. Moreover, vascular invasion is a poor indicator of long-term survival[110]. In one study, risk

factors for early recurrence (within 2 years after surgery) were non-anatomical resection, microscopic vascular invasion, and AFP ⩾ 32 ng/mL[111]. Another retrospective study confirmed the association between the type of surgical approach and the outcome, showing that the cumulative survival rate was significantly higher after anatomical resection compared to non-anatomical resection[112].

As reported above, one crucial issue is the determination of the adequate liver remnant volume after hepatectomy. In normal livers, it is important to preserve the 20%-40% of the TLV or the standard liver volume (SLV)[113-120]. Anderson *et al* suggested that the smallest adequate liver remnant volume should be ⩾ 20% of the SLV in patients with no underlying chronic liver disease[114,121]. However, HCC usually develops in patients with chronic hepatitis or cirrhosis, who are at risk of hepatic failure in case of insufficient liver remnant volume after hepatectomy. In this setting, portal vein embolization (PE) may prevent hepatic failure, because the portal vein branches are blocked to induce compensatory hypertrophy in the remnant liver area[122]. Three-dimensional CT scan allows an accurate determination of the position of major blood vessels and the tumor, as well as resection margins, and liver remnant volume[123].

Perioperative complications include bile leakage, hemorrhage and intra-abdominal abscesses[124,125]. Intraperitoneal drainage is necessary for monitoring and treatment of these complications, even if the Center for Disease Control and Prevention guidelines do not recommend routine drainage in elective hepatectomy. If drainage is required, a closed suction drain should be used and placed through a separate incision distant from the operative one. Moreover, the drainage should be removed as soon as possible[126]. These recommendations have been validated in several studies[127-133]. Moreover, in a randomized clinical trial, subcutaneous drainage was not effective in preventing surgical site infections[134]. Hepatic failure and disseminated intravascular coagulation are other postoperative complications. In one study, the authors evaluated the efficacy of steroids to improve liver function after hepatectomy[135]. They found that serum bilirubin levels were significantly lower in the steroid group on post-operative day (POD) 2 compared with the non-steroid group. The postoperative time courses of bilirubin, IL-6 and the C-reactive protein level were significantly lower whereas the prothrombin level was significantly higher in the steroid arm. No differences in the proportion of patients with complications and the length of hospital stay were reported between the two groups. To unify the definition of post-hepatectomy liver failure (PHLF), the International Study Group of Liver Surgery proposed defining PHLF as an increased international normalized ratio and concomitant hyperbilirubinemia on or after POD 5[136]. PHLF seems to predict the incidence of complications and mortality better than the 50-50 criteria [*i.e.* prothrombin time (PT) < 50% and serum bilirubin > 50 mmol/L][137] and MELD score[138].

Liver transplantation

Liver transplantation has become a feasible alternative for many patients with HCC, given the advances in surgical techniques and immunosuppression.

In 1996, Mazzaferro *et al*[139] defined the so-called Milan criteria, which identified as eligible for transplantation patients with solitary lesions < 5 cm in diameter and those with up to 3 lesions, each one < 3 cm in diameter. Similar survival rates in patients with tumors < 3 cm have been reported by the Bismuth group[140]. The Milan criteria have been accepted worldwide to identify patients which can be safely tranplanted. The limited number of available organs is the main limitation for this procedure. Yao *et al*[141,142] demonstrated that patients with a single lesions ⩽ 6.5 cm, or up to three lesions each one ⩽ 4 cm with a cumulative diameter ⩽ 8 cm had surgical outcomes similar to those transplanted on the basis of Milan criteria. Tumor histology has an important impact on post-transplantation survival, with better outcome in patients with well-differentiated tumors[143,144]. The availability of transplantable grafts remains the critical issue for all patients awaiting liver transplantation, considering that time is a major determinant of overall survival[145-153]. Living donation can be a good choice for transplantation in patients with HCC because the transplant can be planned with an optimal timing to both assess the tumor aggressiveness and minimize the risk of recurrence[154-160]. Another factor that can affect the risk of recurrence after transplantation is the use of immunosuppressive agents. Sirolimus, a bacterial macrolide with immunosuppressive and antineoplastic properties, which inhibits IL-2-mediated lymphocyte proliferation, seems to decrease metastatic tumor growth and angiogenesis in the liver. It was demonstrated that the administration of post-transplant sirolimus, within a steroid-free protocol and a low tacrolimus target, was associated with decreased risk of tumor recurrence and no significant increase in the risk of infection and hepatic artery thrombosis[161-165].

Non-surgical management

Among non-surgical approaches, percutaneous ethanol injection (PEI), microwave ablation (MWA) and percutaneous radiofrequency ablation (RFA) represent the three most widely used techniques for the treatment of HCC less than 5 cm in diameter and/or with less than 3 tumoral lesions.

In RFA, electrical current is applied *via* an electrode resulting in resistive heating and tissue hyperthermia[166]. Tissues adjacent to the electrode are the most effectively heated[167-169]. The mechanism of cytotoxicity in RFA depends on tissue impedance,

with power deposition hindered in regions of high tissue impedance, such as the surrounding lung or tissue adjacent to the electrode, that has undergone water vaporization due to rapid heating[166,169]. Multiple engineering designs have been developed to overcome the limitations caused by tissue impedance, including multi-tined electrodes to expand the contact surface area, saline injection, and internal cooling. Moreover, RFA requires the placement of grounding pads on the patient to close the electrical circuit, and skin burns related to the pads have been reported[170,171]. However, in clinical practice skin burns are rare, considering that larger grounding pads are usually used to improve the dispersion of thermal energy[172]. RFA efficacy may be limited by the "heat sink" effect, consisting in heat dissipation resulting from blood flow. This effect is more marked for lesions close to the liver hilum[173]. There are several reports on RFA use in both primary and metastatic liver tumors. In a Cochrane database analysis, including 11 randomized clinical trials, Weis *et al*[174] analyzed a total of 1819 participants with HCC with the primary outcome of overall survival, comparing RFA to hepatic resection[175-177], PEI[178-183], MWA[184], and percutaneous laser ablation (PLA)[185]. The authors concluded that hepatic resection was superior to RFA in terms of survival, even if RFA might be associated with fewer complications and shorter hospital stay. Moreover, RFA was associated with better survival than PEI, whereas there was no evidence of significant differences between RFA and MWA or PLA.

In the study by Lee *et al*[186], patients undergoing surgical resection were younger and had better liver function reserve and PS than those receiving RFA. When accounting for these differences using propensity score analysis, RFA was superior to surgery for patients with small HCC and Child-Pugh Turcotte score of 5.

MWA relies on the direct application of an electromagnetic field, which causes dielectric hysteresis, leading to local tissue hyperthermia[187]. MWA is able to penetrate through several tissues, including those with high impedance[166,187]. High tissue temperatures can be achieved with MWA, with increased efficacy as compared to RFA[188]. Given the efficacy profile and the shorter time required to achieve ablation, the use of MWA has gradually increased for the treatment of both primary and metastatic tumors of the liver. Ding *et al*[189,190] studied 198 patients with HCC, all in BCLC Stage A meeting Milan criteria and did not find any difference between RFA and MWA in terms of disease-free survival, cumulative survival, and complication rates. Similar results have been reported in other cohorts[184,191].

PEI involves the direct instillation of ethanol into tumors, which results in coagulative necrosis. The technique is relatively simple and inexpensive. However, in clinical practice PEI is limited by poor and irregular distribution of ethanol within the tumor and diffusion into the adjacent normal tissues. Even if some studies with PEI reported favorable outcomes after a long-term follow up (greater than 15 years), most evidences suggest that RFA is associated with better overall survival than PEI[192-194].

The use of external beam radiation therapy (SBRT) in treatment of liver tumors has been traditionally limited by the overall low tolerance of liver tissue to radiation[195]. In fact, radiation produces tumoral killing by transferring energy within atoms, determining the generation of reactive oxygen species with subsequent direct and indirect DNA and cellular damage. The final step is the generation of double-strand DNA breaks, leading to tumor cell death. Radiation can achieve excellent tumor control when delivered to ablative doses[196]. Maximum dose is limited by the radiation tolerance of the surrounding normal liver tissue and adjacent organs. Particularly, radiation-induced liver disease is a complication typically manifesting with the triad of anicteric hepatomegaly, ascites, and elevation of alkaline phosphatase. Imaging techniques, breathing motion control and advances in radiation machines technology permit accurate localization of hepatic tumors and help directing radiation to the tumor while minimizing exposure of surrounding normal liver[197,198]. The size and number of lesions that can be targeted, as well as the radiation dose that can be delivered, depends on normal liver reserve and estimated risk of liver complications. As expected, patients with reduced liver function require dose reduction[199]. Similarly, patients with Child Pugh class B cirrhosis may require dose reduction, while those with Child Pugh class C cirrhosis are not usually eligible for this type of treatment.

Another radiation-based technique is high-dose rate (HDR) CT-guided interstitial brachytherapy[200-202]. Radiation is delivered using an iridium-192 source as a single fraction. The advantage of this technique is a greater protection of the surrounding healthy liver compared to external radiation techniques. A prospective phase Ⅱ trial[203] showed encouraging results for patients with large tumors near the hilum, using average dose of 17 Gy. Mearini *et al*[204] reported favorable outcomes of 35 patients with HCC (tumor size 5-12 cm), treated with HDR brachytherapy. At 12 mo, local control was 93% and no major toxicity was reported.

High-intensity focused ultrasound (HIFU) incorporates multiple ultrasound beams produced by piezoelectric or piezoceramic transducers directed into a three-dimensional focal point[205]. Ultrasound beams are both thermally ablative and cause cavitations to the underlying tissues. Coupling of the ultrasound source and the patient is achieved through a degassed water bath in order to have minimal reflection or absorption of the soundwaves prior to reaching the focal point. The patient is required to minimize movements during the procedure and the focal zone is shifted step by step to cover the area of interest for ablation. The safety and efficacy of HIFU was evaluated in several

studies[206-212]. Ng *et al*[208] reported on a series of 49 patients with HCC (median tumor size 2.2 cm, range, 0.9-8 cm) and concluded that HIFU was effective for those who were not surgical candidates. He reported 1- and 3-year overall survival rates of 87.7% and 62.4%, respectively[209]. Similar data were published by Wu *et al*[210], with overall survival rates of 86.1%, 61.5%,and 35.3% at 6, 12, and 18 mo, respectively. Cheung *et al*[205] reported on the outcomes of HIFU for the treatment of HCC before liver transplantation in 10 patients as compared to 29 patients who received transarterial chemoembolization and found that HIFU was effective (90% had complete response, 10% partial response), with none of the patients on the liver transplant list (n = 5) dropping out[206].

Irreversible electroporation (IRE) is an apparently non-thermal technique in which the direct placement of electrodes creates a pulsed direct current, inducing cytotoxicity in tumor cells by altering transmembrane potentials, which irreversibly disrupt cell membrane integrity[213]. IRE requires the position of at least two applicators in parallel to create ablation zones in the range of 1.5-2 cm per electrode pair[214]. The zone of ablation created by IRE is dependent on multiple factors[213,215], such as electrode spacing and relative position, active tip length, pulse number and duration, and applied voltage. Because of these factors, IRE results more technically challenging than other locally ablative techniques. Moreover, the current generated by IRE causes whole-body muscle contractions and general anesthesia, requiring the use of neuromuscular blockage. In addition, IRE can induce cardiac arrhythmias, though this complication can be avoided with the use of cardiac synchronization of the administered pulses to the complete refractory period of the cardiac cycle[216]. IRE has a theoretical safety advantage as compared to other locally ablative techniques in the treatment of tumors close to structures susceptible to thermal injury, such as major bile ducts. In addition, because of the reduction in the "heat-sink" effect, IRE is potentially more effective for tumors next to major vessels, especially for smaller lesions, and showed excellent local tumor control at 3-6 mo, but high recurrence rates after 12-18 mo[215,217-220].

Cryoablation involves the direct application of a cryoprobe into the tumor. The thermal contact with the tumor results in ice-crystal development and osmotic shock. One recognized advantage of cryoablation is that the zone of ablation is readily visible ("iceball") using CT scan, US, or MRI monitoring, allowing for precise targeting of the ablation area[221]. Moreover, multiple probes can be used simultaneously to create larger ablation zones and shorten procedural times. Despite the technical advantages of cryoablation, its use has been limited by the safety profile. Cryo-shock is an uncommon but potentially life-threatening complication, characterized by thrombocytopenia, acute renal failure, adult respiratory distress syndrome and disseminated intravascular coagulopathy[221]. In a meta-analysis comparing cryoablation to RFA in the treatment of unresectable HCC[222], RFA was superior, particularly in terms of complication rates and local tumor recurrence.

Percutaneous laser ablation (PLA) involves the direct deposition of laser light *via* fiber-optic applicators to induce tissue hyperthermia in tumors. The thin flexible fiber-optic delivery fibers allow for safer and technically easier approaches to tumors[223]. Moreover, feedback and dose-planning systems allow a good control of ablative zones and consequently low complication rates. However, it has been suggested that PLA has some limitations in achieving complete tumor ablation as compared to other locally ablative therapies[185,224,225].

HCC is preferentially supplied by the hepatic arterial inflow, while the normal parenchyma is largely supplied by the portal vein. The trans-arterial chemo-embolization (TACE) procedure is based on these blood supply dynamics. TACE consists in the placement of an intra-arterial catheter in the vessels supplying the tumor, to deliver high concentrations of a chemotherapeutic agent (*e.g.*, doxorubicin, cisplatin or mitomycin) along with an embolic agent, such as lipiodol gelatin sponge or polyvinyl alcohol particles, in order to achieve both targeted chemotherapy and reduction in arterial supply to the tumor.

Drug eluting beads TACE (DEB-TACE), are becoming largely popular because of the favorable safety profile. DEB-TACE delivers small beads, which have been saturated for several hours with chemotherapeutic drugs. The beads occlude the feeding vessels of HCC, while doxorubicin is progressively released, increasing chemotherapeutic concentrations locally and creating tumor necrosis. The choice of bead size, from 75 to 700 μm, depends on tumor size and the preferred level of concentration within the treated volume. The best results are achieved when chemoembolization is performed selectively to segmental or subsegmental arteries feeding the tumor[226]. TACE is considered the standard of care for intermediate stage HCC without vascular invasion or metastases. In several randomized controlled trials, TACE was associated with partial response in 15%-62% of patients, and improved survival[227-233]. Some studies have suggested that complete tumor ischemia may stimulate angiogenesis, resulting in an increased susceptibility to tumor growth rather than suppression. It has been therefore suggested to maintain arterial patency both to prevent this pro-angiogenic effect and to permit repeated treatments[234-237]. Side effects associated with both DEB-TACE and TACE include nausea, vomiting and right upper quadrant pain (post-embolization syndrome), doxorubicin-related cardiac toxicity, bone marrow aplasia, hepatic abscesses, cholecystitis[229,231,238]. Two randomized controlled trials demonstrated improved side effect profiles[239,240] with equivalent survival rates[235,239] and longer time to progression for DEB-TACE in comparison with conventional TACE[240]. A

meta-analysis showed comparable tumor response rates[241].

Radioembolization is a modestly invasive, fluoroscopically guided and microcatheter-based technique, using either yttrium-90 (Y-90) embedded non-biodegradable glass microspheres (25 ± 10 μm) or Y-90 embedded non-biodegradable glass resin-based microspheres (29-35 μm). Radioembolization exploits the preferential arterial blood supply of HCC by delivering radiotherapy directly to the tumor and preserving the normal liver parenchyma. In the target lesion, Y-90 delivers tumoricidal doses of a pure high-energy beta emitter. Because of the short tissue penetration and half-life, Y-90 is an ideal radioisotope for intra-arterial radiotherapy. Patients who have intermediate/advanced BCLC stage HCC and who are not candidates for TACE due to portal vein invasion are ideally candidate to radioembolization with Y-90[242-244]. Radioembolization represents a suitable alternative to chemotherapy for patients with advanced HCC[245]. Moreover, Y-90 radioembolization can be proposed as a bridge to liver transplantation[139,246,247].

As for combinations therapies, one of the most studied approaches is represented by the association of RFA plus TACE. In fact, the decreased blood flow due to TACE reduces heat loss and improves the RFA margins. On the other hand, TACE enhances nearby control of satellite lesions[248]. Several meta-analyses have found that the combination of RFA and TACE is associated with improved survival in comparison with RFA alone, particularly for tumors larger than 3 cm in diameter[249-252]. Hyperthermia is able to potentiate the cytotoxic effect of radiation[253,254]. Additionally, in animal studies, the combined use of radiation and RFA resulted in improved tumor growth control compared with RFA alone[255,256]. The combination of thermal ablation with SBRT represents another encouraging option, even if more research is required to establish the most appropriate dosing and timing regimen[257].

Sorafenib is a small-molecule multikinase inhibitor, which blocks Raf kinase, VEGF receptor and platelet derived growth factor receptor (PDGFR). In two randomized, double-blinded, controlled, phase Ⅲ clinical trials, the SHARP (Sorafenib HCC Assessment Randomized Protocol trial) and the Asia-Pacific (conducted in the Asia-Pacific region), sorafenib was associated with improved progression-free and overall survival in patients with advanced unresectable HCC[258] and currently represents a therapeutic option for patients who are not candidates for curative treatment or TACE.

CONCLUSION

HCC is a major global public health problem due to the rising incidence and high mortality in both developing and developed countries. An important point to be addressed is the promotion of preventive strategies, such as hepatitis B vaccination, and chronic hepatitis B and C treatment, in order to cut down the number of patients who may develop cirrhosis and potentially progress to HCC. Early diagnosis is crucial for curative treatments. As a consequence, patients at risk of developing HCC should be regularly followed up to diagnose HCC in the initial stage. Surgical resection, RFA and liver transplantation are considered the cornerstones of curative therapy, while for patients with more advanced HCC recommended options include sorafenib and TACE.

Unfortunately, most evidence comes from case series and retrospective studies. There is a need for larger, multicenter, randomized studies in order to define the most appropriate, evidence-based therapeutic approach to patients with HCC.

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TOPIC HIGHLIGHT

2015 Advances in Hepatocellular Carcinoma

Nanoparticles for targeted delivery of therapeutics and small interfering RNAs in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the 5th most common malignancy which is responsible for more than half million annual mortalities; also, it is the third leading cause of cancer related death. Unfavorable systemic side-effects of chemotherapeutic agents and susceptibility to the degradation of small interfering RNAs (siRNAs), which can knock down a specific gene involved in the disease, have hampered their clinical application. So, it could be beneficial to develop an efficient carrier for the stabilization and specific delivery of drugs and siRNA to cells. Targeted nanoparticles have gained considerable attention as an efficient drug and gene delivery system, which is due to their capability in achieving the highest accumulation of cytotoxic agents in tumor tissue, modifiable drug pharmacokinetic- and bio-distribution, improved effectiveness of treatment, and limited side-effects. Recent studies have shed more light on the advantages of novel drug loaded carrier systems *vs* free drugs. Most of the animal studies have reported improvement in treatment efficacy and survival rate using novel carrier systems. Targeted delivery may be achieved passively or actively. In passive targeting, no ligand as homing device is used, while targeting is achieved by incorporating the therapeutic agent into a macromolecule or nanoparticle that passively reaches the target organ. However, in active targeting, the therapeutic agent or carrier system is conjugated to a tissue or cell-specific receptor which is over-expressed in a special malignancy using a ligand called a homing device. This review covers a broad spectrum of targeted nanoparticles as therapeutic and non-viral siRNA delivery systems, which are developed for enhanced cellular uptake and targeted gene silencing *in vitro* and *in vivo* and their characteristics and opportunities for the clinical applications of drugs and therapeutic siRNA are discussed in this article. Asialoglycoprotein receptors, low-density lipoprotein, ganglioside GM1 cell surface ligand, epidermal growth factor receptor receptors, monoclonal antibodies, retinoic acid receptors, integrin receptors targeted by Arg-Gly-Asp peptide, folate, and transferrin receptors are the most widely studied cell surface receptors which are used for the site specific delivery of drugs and siRNA-based therapeutics in HCC and discussed in

detail in this article.

Key words: Small interfering RNA; Targeted delivery; Nanoparticle; Hepatocellular carcinoma; Chemotherapeutic agents

Core tip: Targeted nanoparticles have gained considerable attention as an efficient drug and gene delivery system in hepatocellular carcinoma owing to their capability for achieving the highest accumulation of cytotoxic agents in tumor tissue, modifiable drug pharmacokinetic- and bio-distribution, improved effectiveness of treatment, and limited side-effects. This review covers a broad spectrum of targeted nanoparticles as therapeutic and non-viral small interfering RNA (siRNA) delivery systems, which are developed for enhanced cellular uptake and targeted gene silencing *in vitro* and *in vivo*. Their characteristics and opportunities for the clinical applications of drugs and therapeutic siRNA are discussed in this article.

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EPIDEMIOLOGY

Hepatocellular carcinoma (HCC) as the 5th most common malignancy is the most common primary liver cancer and is responsible for more than half million annual mortalities, which makes it the third leading cause of cancer related deaths[1]. This disease more dominantly affects males than females, the ratio of which is usually around 3:1 or 4:1 in most populations[2]. At the moment, HCC is most prevalent in East Asia; but, it is rapidly pervading through most of the Western nations and the number of diagnosed patients is rapidly increasing[1,3].

Etiology

Several risk factors are held responsible for the occurrence of HCC, but with varying importance levels in different regions. This disease mostly occurs in persons with a history of other liver diseases such as cirrhosis, which is the unique nature of HCC and points to the most important risk factors as Hepatitis C and B as well as alcoholic and non-alcoholic fatty liver. Other important risk factors are toxic exposure to aflatoxins and vinyl chloride, diabetes mellitus, obesity, diet, hemochromatosis, Wilson's disease, type 2 diabetes, hemophilia, and genetic factors[4]. The overall average 5-year survival rate of HCC is estimated as 70% for the patients undergoing surgery[5].

SIGNS AND SYMPTOMS

There are several staging systems for HCC which determine the course of treatment and treatment prognosis[6]. HCC patients may show jaundice, bloating from fluid in the abdomen, easy bruising from coagulopathy, loss of appetite, unintentional weight loss, nausea, vomiting, or fatigue. Patients usually complain about right upper quadrant pain, weight loss, and deterioration of liver function in cirrhotic cases. Most symptoms are unspecific such as abdominal pain, malaise, fever, jaundice, and anorexia. Ascites, hemorrhage, and encephalopathy may also occur; but, a large number of population may remain asymptomatic[7].

DIAGNOSIS

HCC screening is recommended for high risk patients and the most frequently used surveillance methods are testing serum α-fetoprotein (AFP) and abdominal ultrasound in 6 mo intervals[8,9]. Ultrasound is often the first imaging and screening modality which is used. In the patients with higher suspicion of HCC (such as rising alpha-fetoprotein and des-gamma carboxyprothrombin levels), the best method of diagnosis involves a computed tomography (CT) scan of the abdomen using intravenous contrast agent. A biopsy is not needed to confirm the diagnosis of HCC if certain imaging criteria are met. An alternative to a CT imaging study would be magnetic resonance imaging (MRI)[8,9].

MANAGEMENT AND TREATMENT MODALITIES FOR HCC

Non-medicinal managements of HCC

If benefits outweigh surgery risks, the patient is a candidate for surgical modalities such as liver resection in the case of early-stage non-cirrhotic patients and liver transplantation in the case of chronic disease and cirrhosis. However, early-stage HCC patients that are not qualified for surgical interventions can alternatively benefit from minimally invasive treatments such as percutaneous ablation and intermediate-stage HCC cases that have not shown vascular invasion and cancer related symptoms could undergo trans catheter arterial chemoembolization (TACE), which is usually performed for unresectable tumors or as a temporary treatment while waiting for liver transplant[10]. Other managements include: interventional radiology; http://en.wikipedia.org/wiki/File:HepatoCellular_Ca.JPG; radiofrequency ablation (RFA) using high frequency radio-waves to destroy tumor by local heating; selective internal radiation therapy (SIRT)

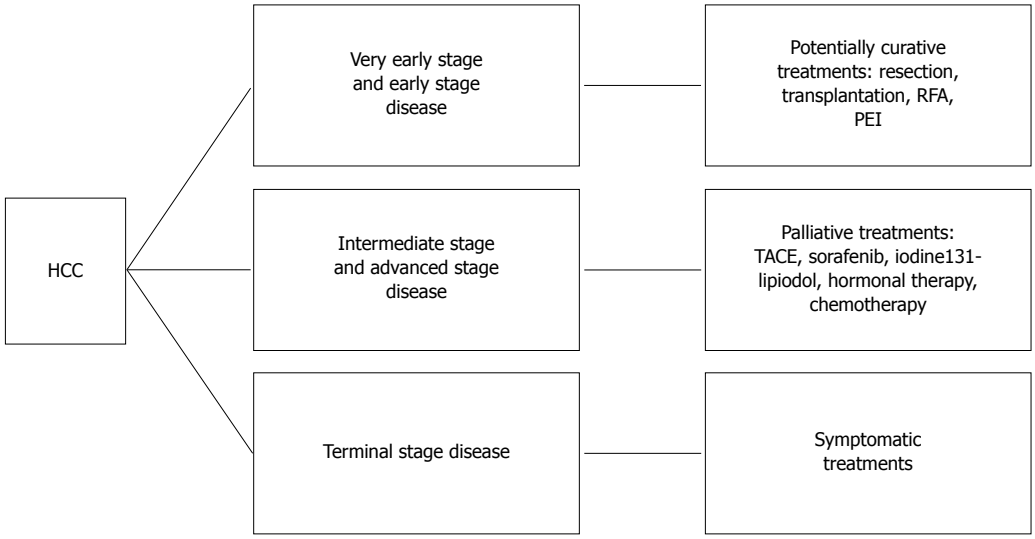

Figure 1 Schematic representation of various treatment strategies for hepatocellular carcinoma at different stages according to the Barcelona Clinic Liver Cancer algorithm. RFA: Radiofrequency ablation, PEI: Percutaneous ethanol injection; TACE: Trans catheter arterial chemoembolization.

used to destroy the tumor and thus minimize exposure of healthy tissue; intra-arterial iodine-131-lipiodol administration and percutaneous ethanol injection (PEI) as well-tolerated methods; combined PEI and TACE for the tumors of larger than 4 cm in diameter; and portal vein embolization as a method using a percutaneous trans hepatic approach in which an interventional radiologist embolizes the portal vein supplying the side of the liver and the tumor. There are currently two products of embolic microspheres: SIR-Spheres and Thera Sphere. The latter is an FDA approved treatment for primary HCC. SIR-Spheres are FDA approved for the treatment of metastatic colorectal cancer, but outside the United States. Cryosurgery is also another management of HCC that is a new technique and can destroy tumors in the liver. High intensity focused ultrasound is another therapeutic treatment of HCC[10]. Figure 1 summarizes various treatment strategies for HCC at different stages according to the *Barcelona Clinic Liver Cancer* algorithm.

Therapeutics of HCC

Palliative and restricted therapy to a localized region of the body could also be used in intermediate- to advanced-stage patients, for whom embolization is not feasible. Some examples of locoregional therapy methods are internal radiation and hormonal therapy including antiestrogen therapy with tamoxifen (usually considered ineffective), octreotide (somatostatin analogue), and adjuvant chemotherapy. No randomized trial has shown the benefit of neoadjuvant or adjuvant systemic therapy in HCC. Single trial has shown a decrease in new tumors among the patients receiving oral synthetic retinoid for 12 mo after resection/ablation. Results have not been reproduced[11].

Since systemic chemotherapy is not proved to significantly increase survival rate in HCC patients and due to the overall side-effects of chemotherapeutic agents, their dose limitation, and possibly the expression of multi drug resistance gene (MDR-1) in HCC, chemotherapy is now considered one of the palliative therapies for HCC, while the survival rate of incurable HCC patients remains poor[12].

Doxorubicin is one of the most frequently used cytotoxic agents for the chemotherapy of non-resectable HCC tumors, even though it has high toxic side-effects and has shown no increased survival rate[13]. Randomized phase Ⅱ and Ⅲ studies have also compared the response rates of doxorubicin *vs* the frequently used PIAF regimen consisting of cisplatin/interferonα-2b/doxorubicin/5-fluorouracil; while the response rates have been slightly enhanced, the survival rates are not significantly improved[14,15]. Gemcitabine has been found to be more effective in hepatic cancers and the combination regimen of gemcitabine and oxaliplatin (GEMOX) along with bevacizumab has slightly increased the survival time in a phase Ⅱ study[16].

Studies on interferon-α (IFN-α) immunotherapies have suggested that IFN-α could have survival benefits for non-curable HCC patients when used in combination with other agents[17].

Many molecular targeted therapies are also under phase Ⅱ and Ⅲ studies. A receptor tyrosine kinase inhibitor, sorafenib, as an FDA approved drug, may be used in the patients with advanced HCC. Sorafenib is a small molecule that inhibits tumor-cell proliferation and tumor angionesis. It correspondingly increases the rate of apoptosis in other tumor models. Sorafenib is one of the molecular targeted small molecule agents, which blocks vascular epithelial growth factor receptors (VEGFRs) 1, 2 and 3 and platelet derived growth factor receptor β (PDGFR-β) through multikinase inhibition and leads to the inhibition of tumor growth and

angiogenesis[18,19]. Results of meta-analysis based on phase Ⅱ and Ⅲ trials have suggested that sorafenib-based chemotherapy is superior to placebo-based chemotherapy in terms of overall survival without considerable increase in toxicity[20].

Bevacizumab is another molecular drug which is, in fact, a humanized monoclonal antibody against VEGF and, hence, results in the blockade of angiogenesis. Findings of phase Ⅱ clinical trials have suggested that bevacizumab might increase the survival rate of patients with nonmetastatic HCC[21]. Erlotinib and sunitinib are also other small molecules for the inhibition of thyrosine kinase that have been found effective in phase Ⅱ trials[22,23]. Cetuximab and lapatinib are other molecular therapeutics that are currently under phase Ⅱ studies for non-resectable HCC[24].

Small interfering RNA-based treatment of HCC

Small interfering RNA (siRNA) is a double-strand RNA molecule which is also named short interfering RNA or silencing RNA with 20-25 base pairs in length. siRNA interferes with the expression of specific genes with complementary nucleotide sequences. It causes mRNA to get broken down after transcription and result in no translation[25]. RNA interference (RNAi) may represent a powerful strategy to interfere in key molecular pathways involved in cancer and has established a new area of clinical therapy for HCC. siRNA induced RNAi presents an effective and simple method to silence a wide range of cancer-associated genes. A number of siRNAs have been established that are capable of silencing some different types of human HCC gene targets, such as livin, cyclin E, VEGF, COP9 signalosome subunit 5, c-Myc, and so on[26-29]. The most important molecular and biochemical markers of human hepatocellular carcinoma effective in progression and poor prognosis include glypican-3, Dickkopf-1, S100A4, S100A14, SOX6, SUOX, AKR1B10, and CD34, cystine/glutamic acid transporter, GRK6, GPR87, metallothioneins, retinoic acid-induced protein 3, synovial sarcoma X breakpoint 2, protein phosphatase magnesium-dependent 1 delta, BCL9, interferon regulatory factor-1 and 2, CDK4, LASP-1, PTP4A3, fatty acids, PAK5, hnRNPL, cylindromatosis gene, melanoma-associated antigen family protein D-4, EphA3, and Flotillin-1[30]. Knocking down of the genes of these biomarkers by siRNA may be used for the treatment of HCC. Li *et al*[31] presented the development of TetR siRNA therapeutics for HCC using an integrated approach, including the development of an efficient lipid nanoparticle (NP) delivery system, the identification of a robust therapeutic target that does not trigger liver toxicity upon target knock down, and the selection of potent and non-immunogenic siRNA molecules against the target. The TetR-ODC-Luc, HepG2, or HuH7 cells were inoculated into the liver of 6 to 8 wk old severe combined immune-deficient female mice to create various orthotopic HCC liver tumor models. The resulting siRNA-containing lipid NPs produced significant antitumor efficacy in orthotopic HCC models and, thus, represented a promising starting point for the development of siRNA therapeutics for HCC.

Kinesin spindle protein (KSP) plays a critical role in mitosis. Inhibition of KSP function leads to cell cycle arrest at mitosis and, ultimately, cell death. In the study done by Doan *et al*[32], KSP expression was suppressed by specific siRNA in Hep3B cells and evaluated its anti-tumor activity. KSP-siRNA transfection induced apoptosis and could increase chemo sensitivity to DOX in Hep3B cells, even at low doses compared to the control. This method may yield promising results for eradicating HCC cells *in vitro*.

Another important siRNA type in HCC is the specific gene silencing siRNA of AFP which is an oncoembryonal protein highly expressed in the majority of HCCs. AFP may be involved in multiple cell growth regulating, differentiating, and immunosuppressive activities. Effects of AFP gene silencing by siRNA on the apoptosis and proliferation of HCC cell line EGHC-9901, which highly expresses AFP has been investigated. Western blot and RT-PCR assay have demonstrated that siRNA-AFP induces high expression of caspase-3, caspase-8, caspase-9, and Bcl-2[33].

$p28^{GANK}$ is an HCC oncogene. The adenovirus-delivered siRNA (AdsiRNA) is applied to inhibit this oncogene in HCC cell lines and the antitumor effect is investigated. The T7-RNA polymerase system is used to screen the specific target site. AdSiRNA could suppress $p28^{GANK}$ expression by up to 80% in HCC cells. Depletion of $p28^{GANK}$ induces caspase-8- and caspase-9-mediated apoptosis of HCC cells. Finally, targeting $p28^{GANK}$ by adenovirus injection inhibits the growth of established tumors in nude mice. This study shows that the T7-RNA polymerase system screening-based AdSiRNA can be used successfully to silence an oncogene. $p28^{GANK}$ may serve as a novel therapeutic target for treating HCC[34].

NANOPARTICLE-BASED DRUG DELIVERY

Nanoparticulate drug delivery systems are solid, colloidal particles with the particle size of 10 to 1000 nm. However, in nanomedicine, they often refer to devices < 200 nm (*i.e.*, the width of microcapillaries). There are two types of NPs: nanocapsules and nanospheres. Nanocapsules are vesicular systems in which a drug is confined to a cavity surrounded by a polymeric membrane, whereas nanospheres are matrix systems in which the drug is physically and uniformly dispersed. NPs can load the active ingredients in different forms of dissolved, entrapped, adsorbed, attached, and/or encapsulated into or onto a nanomatrix. Systemic unfavorable side-effects of chemotherapeutic agents and other drugs have led to research on the development of new agents or

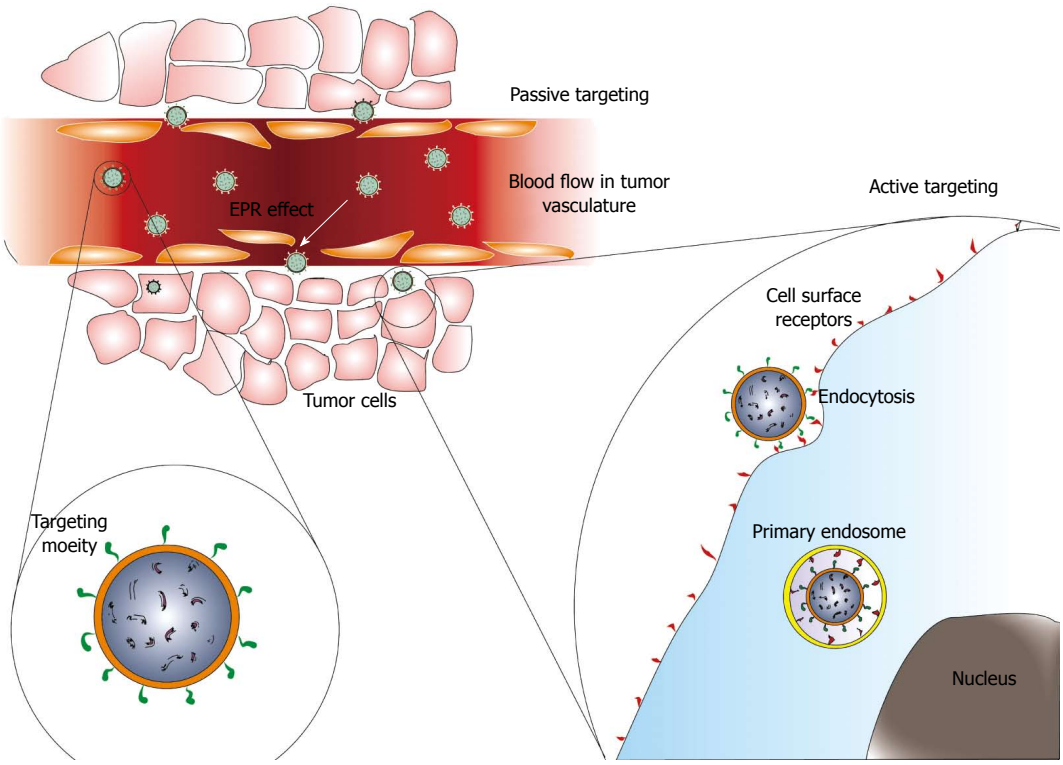

Figure 2 Different methods of targeted delivery of nanoparticles by passive or active mechanism. EPR: Enhanced permeation and retention.

therapeutic strategies. The main objectives are to achieve the highest accumulation of cytotoxic agents in tumor tissue, modify drug pharmacokinetic- and bio-distribution, improve effectiveness of treatment, and limit the side-effects.

Targeted delivery may be achieved passively or actively (Figure 2). In the passive targeting, no ligand is used as a homing device and targeting is achieved by incorporating the therapeutic agent into a macromolecule or nanoparticle that passively reaches the target organ. In this strategy, the leaky nature of vessels in cancer tissue and lack of well-defined lymphatic system can enhance the permeation and retention of NPs, which is called the enhanced permeation and retention (EPR) effect. However, in active targeting, the therapeutic agent or carrier system is conjugated to a tissue or cell specific receptor which is over-expressed in special malignancy using a ligand called homing device[35].

CELL SURFACE RECEPTORS IN HCC

HCC has one of the worst prognoses for survival as it is poorly responsive to both conventional chemotherapy and mechanism-directed therapy. This issue is due to the lack of therapeutic concentration in the tumor tissue coupled with the highly toxic off-site effects exhibited by these compounds. Consequently, the best packaging for the therapy of HCC will involve three components: a potent therapeutic, a rationally designed drug delivery vehicle to enrich the target site concentration of the drug, and a surface ligand that can lead to greater propensity for internalization by tumor cells compared to parenchyma. Before addressing the variety of targeted drug delivery systems used in HCC, an introduction to the different receptors that are over-expressed in this disease and may be used for the active targeting of chemotherapeutic agents loaded in NPs may be useful. Serotonin is a well-known neurotransmitter and vasoactive substance. Recent studies have indicated that serotonin contributes to liver regeneration and promotes tumor growth of human HCC. The serotonin receptors 1B and 2B are expressed, respectively, in 32% and 35% of the patients with hepatocellular cancer. Both receptors are associated with an increased proliferation index in Huh7 and HepG2 cell lines[36].

Gamma-aminobutyric acid and gamma-aminobutyric acid A receptor θ subunit play important roles in HCC development and progression and could be a promising molecular target for the development of new diagnostic and therapeutic strategies for HCC[37].

Over-expression of fibroblast growth factor receptor 3 (FGFR3), which is a signal transduction and cell proliferation related gene in HCC, is another important receptor with an important role in liver carcinogenesis. FGFR3 may be an ideal candidate as a molecular marker in the diagnosis of HCC and a potential therapeutic target[38]. Epidermal growth factor receptor (EGFR) is frequently over-expressed in HCC. EGFR expression has shown borderline association with cirrhosis, but not with other examined clinicopathologic

parameters. EGFR over-expression is present in a majority of HCC types, which suggests a role for EGFR antagonists in therapy[39].

Somatostatin receptor related alterations are potentially novel prognostic and predictive biomarkers for HCC with a special emphasis on the therapeutic potential of somatostatin analogues in HCC management[40].

Insulin receptor (IR) exists as two isoforms resulting from the alternative splicing of IR pre-mRNA. IR-B promotes the metabolic effects of insulin, while IR-A rather signals proliferative effects. IR-B is predominantly expressed in the adult liver. Alternative splicing of IR pre-mRNA is dysregulated in HCC, while it is normal in adjacent non-tumor liver tissue. Increased expression of IR-A during the neoplastic transformation of hepatocytes could mediate some of the adverse effects of hyperinsulinemia on HCC[41].

Prostaglandin E_2 (PGE_2) has been implicated in cell invasion in HCC *via* increased β1-integrin expression and cell migration by activating the PKC/NF-κB signaling pathway. Targeting PGE_2/EP1/PKC/NF-κB/FoxC2/β1-integrin pathway may represent a new therapeutic strategy for the prevention and treatment of this cancer[42]. High levels of adenosine accumulate in hypoxic tissues during the rapid growth of tumors, suggesting that the activation of adenosine receptors may facilitate tumor progress. A_{2b} expression is up-regulated in HCC and its expression level is correlated to tumor progression in HCC, which suggest that A_{2b} may be a novel target for HCC therapeutic strategy[43]. The insulin-like growth factor (IGF) pathway is implicated in the pathogenesis of HCC and may be important in nonalcoholic fatty liver disease. Significant associations have been seen between IGF-1 expression and liver cirrhosis and survival after resection in patients with HCC, independent from their underlying liver disease[44].

Galactosamine-mediated targeted delivery of anti-cancer drugs in the liver has been tested, because its receptor, asialoglycoprotein receptor 1 (ASGPR1), is expressed in the liver and not in other human tissues. Mammalian hepatic ASGPRs mediate the binding, internalization, and degradation of extracellular glycoproteins with exposed terminal galactose, lactose, or N-acetyl-galactosamine residues[45].

Androgen (AR) signaling has also been shown to suppress the metastasis of HCC among the patients with late-stage disease. In addition, there is evidence that therapy comprising Sorafenib and agents that enhance the functional expression of AR may suppress the progression of late-stage HCC[46].

Another surface receptor which is over-expressed in HCC is the folate receptor (FR). Folate is a basic component of cell metabolism and DNA synthesis and repair. Rapidly dividing cancer cells have an increased requirement for folate to maintain DNA synthesis, an observation supported by the widespread use of anti-folates in cancer chemotherapy. FR levels are high in specific malignant tumors of epithelial origin compared to normal cells and are positively associated with tumor stage and grade, which raises the questions of its role in tumor etiology and progression. Although the precise mechanism of pathway(s) for FR uptake has not been exploited, phagocytosis is proposed from the observation that FR recycles between an acid-resistant (intracellular) and acid-sensitive (extracellular) pool. GPI-anchored proteins are diffusely distributed at the cell surface and it is proposed that FR is cross-linked by these proteins and then concentrated in clusters at the cell membrane surfaces called Caveolae, whereby the membrane would transiently close and internalize the folate-bound receptor complex. When the internal compartment of the cell shows increased acidification folate is separated from the receptor and using the energy generated from the acidic gradient moves across the membrane into the cytoplasm of the cell, then, the next cycle would be started at the cell surface membrane by the exposure of the receptors[47].

Transferrin receptor (TfR) is also over-expressed in many malignant cells, including breast cancer, pancreatic cancer, prostate cancer, colon cancer, lung cancer, and leukaemia[48-50] cells. It is also over-expressed in some cell lines of HCC including HepG2, J5, Bel-7402, Huh7, and SK-Hep-1. It is a carrier protein for Tf, imports iron into the cell by internalizing the Tf-iron complex through receptor-mediated endocytosis, and is regulated in response to intracellular iron concentration[51].

Retinoid analogues have been reported to inhibit the growth of HCC. Sano *et al*[52]'s study showed that retinoic acid receptor-alpha is the dominant receptor in HCC, which suggests that selective retinoid analogues of this receptor may be useful for chemotherapy.

TARGETED NANOPARTICLE-BASED DRUGS AND SIRNA DELIVERY IN HCC

In different works aiming to reduce undesirable side-effects of therapeutic agents in non-target organs, scientists have tried to sign up one of these over-expressed receptors for the active targeting of drugs to HCC by the conjugation of a specific ligand to the NPs which fit to the receptor and facilitate the endocytosis of the drug loaded carrier to the cells. In the following sections, it is attempted to illustrate the potentials of novel strategies based on targeted nanoparticulate delivery systems used in the treatment of HCC incorporating drugs or siRNA through different over-expressed cell surface receptors. Table 1 summarizes some of the different targeted nanocarriers used for the delivery of therapeutic agents and in HCC, while Table 2 shows the reported delivery systems of targeted NPs for siRNA in this disease.

Passive targeting by pegylated NPs

As mentioned before, targeted drug delivery may be achieved by EPR effect *via* passive targeting. For this purpose, it is necessary for the carrier to have

Table 1 Summary of studied targeted nanoparticles used *in vitro*/*in vivo* for drug delivery in hepatocellular carcinoma using different targeting moieties

Type of nanocarrier	NPs composition	Targeted for receptor	Active moiety	Specific Remarks	Ref.
Synthetic or natural polymeric NPs	Polyethylenglycol (PEG), dithiodipropionate, hyaluronic acid	Asialogycoprotein receptor (ASGPR) , no receptor (EPR effect)	Doxorubicin (DOX)	The NPs were sensitive to acidic environment of endosomes and high intracellular Glutathion concentrations	[62]
Synthetic or natural polymeric NPs	poly(caprolactone), PEG	no receptor (EPR effect)	Docetaxel	The NPs had a long circulating characteristic and longer retention time in tumor cells	[63]
Synthetic or natural polymeric NPs	Galactose (GA), chitosan,	ASGPR	5-FU	-	[66,74,75]
Synthetic or natural polymeric NPs	O-carboxymethyl chitosan, GA	ASGPR	Paclitaxel (PTX)	-	[68]
Synthetic or natural polymeric NPs	Hyaluronic acid (HA), GA	ASGPR	PTX	The dual targeting allows for the NPs to get internalized by tumor cells more specifically	[69]
Synthetic or natural polymeric NPs	Hematoporphyrin, Bovine serum albumin (BSA)	LDL	DOX	The photosensitizing properties of hematoporphyrin allowed for an accurate monitoring of NP uptake by imaging	[88]
Synthetic or natural polymeric NPs	Heat-liable enterotoxin subunite B (LTB), BSA	Ganglioside GM1 receptor	5-FU	-	[92]
Synthetic or natural polymeric NPs	Galactosylated chitosan, polycaprolactone	ASGPR	Curcumin	-	[72]
Synthetic or natural polymeric NPs	Galactosylated chitosan, mPEG-SH	ASGPR	Norcantharidin (NCTD)	The drug was actually loaded using ionic cross linkage between NCTD and chitosan	[76]
Synthetic or natural polymeric NPs	SM5-1, PLA	Membrane antigens	5-FU	-	[96]
Synthetic or natural polymeric NPs	Apotransferin, BSA	transferrin receptor	DOX	-	[138]
Synthetic or natural polymeric NPs	Apotransferrin, Lactoferrin	Transferrin receptor	DOX	-	[139]
Synthetic or natural polymeric NPs	Chitosan, retinoic acid (RA), Albumin	RA receptor	DOX	-	[105]
Mixed NPs	GA, DOX, Alginate	ASGPR	DOX	-	[70]
Mesoporous high surface area silica core fused to a liposome (protocell)	Silica, PEG, Zwitterionic lipids, phosphatidylethanolamine	SP94 receptor, no receptor (EPR effect)	DOX	The fusogenic peptide used allows for more efficient internalization. The nanoporous silica core also gives the NPs, a significantly higher surface area	[64]
Nano micelle	PEG, polycaprolactone, SPION, folate	Folate receptor	Sorafenib	The SPION loaded NPs could be efficiently monitored using MR imaging	[128]
Nano micelle	RGD, PEG, stearic acid, chitosan	Integrins	DOX	-	[112]
Nano liposome	egg phosphatidylcholine, cholesterol, monomethoxy PEG-distearoyl phosphatidylethanolamine, and Lactose - dioleyl phosphatidylethanolamine (DOPE)	ASGPR	DOX	-	[73]
Nano liposome	Anti CD44 antibody, cholesterol, DOPE, DSPC, DSPE-$(PEO)_4$-cRGDfK, DSPE-mPEG	CD44	DOX	-	[97]
Lipid nanocarriers	Lactobionic acid, Stearyl amine, lecithin, glyceryl monostearate, oleic acid	AGPR	5-FU	-	[71]
Magnetic NPs	$FeCl_2$, $FeCl_3$, CMC, EpCAM aptamer	Epithelial cell adhesion molecule	DOX	These magnetic nanoparticles were suggested as a candidate for MR imagine of HCC	[113]
Magnetic NPs	Fe_3O_4/Fe, silica	no receptor (EPR effect)	ABT-888, Temozolamide	The co-delivery of the two drugs both inhibits transcription of survival genes and has cytotoxic effect	[65]
BSA NPs	Glycyrrhizic acid (GA), BSA, 10-hydroxycamptothecin (HCPT)	ASGPR	FITC	-	[67]
Gold NP	Gold, cetuximab	EGFR	gemcitabine	When coupled with RF-hyperthermia, these NPs were significantly effective at tumor growth inhibition	[94]
Nanosuspension	DSPE, PEG, FA, soy lecithin	Folate receptor	Docetaxel	-	[136]
Dendrimer	poly(methacryloyl sulfadimethoxine) (PSD), PEG, Lactose	ASGPR	DOX	-	[77]

Virus-like NP	MS2 capsid, Ricin toxin A chain, SP94, H5WYG fusogenic peptide, PEG	SP94 receptor	DOX, cisplatin, 5-FU	-	[142]
Core-shell NP	Poly(vinyl alcohol), albumin	Transferrin receptor	DOX, sorafenib	The synergistic effect of DOX and sorafenib here increased tumor inhibition more significantly	[141]
Miscellaneous	PLGA, PVA, chitosan, Asialofetuin	ASGPR	EPI	Co-delivery of EPI with tocotrienols as anti-oxidative agents, resulted in significantly lower cardiotoxicity and higher apoptosis level	[85]
Miscellaneous	Benzyl malolactonate, PEG, Fluorescein amine, Biotin, cyclic Biotin-RGD peptide, streptavidin	Biotin Receptor, integrins	DOX	The use of streptavidin for grafting a peptide suggests that a number of peptides could be grafted in NPs without needing any additional chemistry	[115]

NP: Nanoparticle; LDL: Low-density lipoprotein; EPR: Enhanced permeation and retention; FITC: Fluorescein isothiocyanate.

Table 2 Summary of studied targeted nanoparticles used *in vitro*/*in vivo* for small interfering RNA delivery in hepatocellular carcinoma using different targeting moieties

Type of nanocarrier	Active moiety	Targeted receptor	Suggested Mechanism	Ref.
Lipid nanoparticle	TetR siRNA	-	-	[31]
Novel nonocarrier consisting of a silica core fused to liposomes (called protocell)	Model siRNA	SP94 protein and EPR	-	[64]
Galactose mediated trimethylchitosan cysteine NPs	VEGF-siRNA and Survivin shRNA-expression pDNA (iSUR-pDNA)	ASGPR	Silencing of tumor growth genes	[86]
A phospholipid-cholesterol nanocomplex	Pokemon siRNA	LDL receptor	Cell growth inhibition	[89]
A SPION called SilenceMag	Human VEGF siRNA	EGFR	Tumor growth inhibition	[95]
PEGylated Polyethyleneamine SPION	Survivin siRNA	Integrin	Induction of apoptosis	[114]
Virus-like nanoparticle of bacteriophage MS2	Anti-cyclin siRNA	Folic acid receptor, SP94, transferrin receptor	Induction of apoptosis	[142]
Lipid nanoparticle	Integrin b1 siRNA	integrin	Inhibition of proliferation and tumor cell death	[116]

SPION: Superparamagnetic iron oxide nanoparticle; siRNA: Small interfering RNA; LDL: Low-density lipoprotein; EPR: Enhanced permeation and retention; EGFR: Epidermal growth factor receptor; ASGPR: Asialoglycoprotein receptor.

enough time to reach the affected area. Surface-modification of delivery vehicles with polyethylene glycol (PEG), *i.e.*, PEGylation, is a promising method for enhancing *in vivo* stability and performance of various non-viral drug and gene vectors, which results in the production of stealth NPs[53-59]. In addition, PEGylation can dramatically improve particle transport through biological obstacles, such as mucus[60]. The efficacy of nucleus-targeted drug- or gene-carrying NPs may be limited by slow transport through the molecularly crowded cytoplasm following endosome escape. NPs may stick to cytoskeletal elements and cellular organelles may be steric obstacles for the efficient intracellular transport of NPs. Therefore, surface coating of NPs with PEG can potentially reduce the adhesive interactions of colloids with intracellular components[61]. In this attempt, the dual sensitive spherical NPs of PEGylated dithiodipropionate-hyaluronic acid copolymer (PEG-SS-HA) was produced and loaded with DOX by Xu *et al*[62]. This NP is prone to releasing its DOX content in response to the acidic pH of intracellular lysosomes and reduction by high intracellular glutathion (GSH) concentration. Due to PEGylation, NPs have a stealth circulation and are finally uptaken by liver cells because of the HA content. The *in vitro* studies have demonstrated that these NPs are effectively internalized by HepG2 cell line and cause the inhibition of cell growth.

Docetaxel loaded poly ethyleneglycol-poly (caprolactone) (mPEG-PCL) NPs are another example of passive targeted NPs used on hepatic cancer cell line H22 *in vitro.* This study results in the same cytotoxic effect as the free commercial Docetaxel. NPs have a long circulation, higher accumulation inside tumor parenchyma, longer retention time in tumor cells *in vivo,* and hence effective tumor growth inhibition[63].

Pegylation along with peptide targeted NPs have been used for efficient co-delivery of therapeutic and siRNA in HCC, one of which is a spherical, nanoporous high surface area silica core fused to liposomes. The obtained hybrid and supported lipid bilayer, named protocell, is then modified using a targeting SP94 peptide, PEG, and fusogenic peptide. This novel nanocarrier has a much higher surface area that results in higher capacity for therapeutic loading as well as enhanced stability and selectivity compared to the liposomes with the same size. DOX, a low molecular weight model drug, and a siRNA model are loaded into these protocells. The protocells are demonstrated to be internalized rapidly by Hep3B cells and DOX is released in an efficient manner in physiological mimicking environments. The protocells designed here are potential candidates for the delivery of a disparate set of cargoes and therapeutic and siRNA cocktails with

the flexibility of selecting several targeting ligands and other characteristics[64].

Sometimes, passive targeting is potentiated by an external magnetic field to concentrate the NPs in the affected area. An example of this method of targeting is a study in which a poly (ADP-ribose) polymerase 1 (PARP-1) inhibitor (ABT-888) in conjunction with the alkylating agent temozolomide (TMZ) is used as a therapeutic strategy to increase the cytotoxic effect of drug on HCC. Fe_3O_4/Fe core with a silica shell is prepared for the dual delivery of ABT-888 and TMZ. This nanosystem is tested *in vitro* on three tumoral and one non-cancerous cell lines of the liver. An extended release kinetic is achieved and the NPs are accumulated in tumor cells and show higher efficacy in apoptotic cell death compared to free drug[65].

Asialoglycoprotein receptors

Although PEGylation has been widely used to enhance the accumulation of NPs in tumor tissues through EPR effect, it still inhibits cellular uptake and affects intracellular trafficking of carriers. On the other hand, active targeting of molecules displays better cell selectivity and enhances the poor tumor penetration effect. As mentioned before, asialoglycoprotein receptors are another type of receptors over-expressed on HCC cells. They are lectins which bind asialoglycoprotein; glycoproteins from which a sialic acid has been removed to expose galactose residues. The receptors, which are located on liver cells, remove the target glycoproteins from circulation. Glycyrrhetinic acid (GA) is one of the sugar type ligands, which is used for the targeted delivery of NPs to HCC. GA modified chitosan NPs are produced and loaded with 5-FU. The NPs provide a sustained release system that halts tumor cell growth *in vitro* in a time and dose dependent manner. The *in vivo* studies on an ortothopic model of liver cancer in mouse have demonstrated significant tumor growth inhibition and prolonged life span[66].

In another study, glycyrrhizic acid modified NPs of bovine serum albumin are synthesized by different concentrations of each agent and loaded with 10-hydroxycamptothecin. These NPs have higher affinity for hepatic tumor cells and the treatment group shows a higher amount of tumor growth inhibition than the control group[67].

Use of glycyrrhizin modified O-carboxymethyl chitosan NPs loaded with paclitaxel is another example of targeted NPs toward ASGPRs, which are tested on HCC cell line of SMMC-7721. The blank NPs show complete biocompatibility and no toxicity. The cell internalization of glycyrrhizin conjugated NPs is almost 10 times higher than the non-targeted NPs. Furthermore, the tumor growth inhibition is significantly higher than non-targeted NPs and free paclitaxel[68].

A double target nanocarrier containing glycyrrhetinic acid-grafted-hyaluronic acid (GAHA) NPs is also synthesized. This double target nanocarrier against liver tumor cells consist of a hyaluronic acid shell with glycyrrhetinic acid grafts encapsulating paclitaxel and is tested on two different cell lines of human HCC. The uptake of NPs is high in HepG2, which has both receptors for HA and GA. The carrier itself has a low cytotoxicity level and *in vivo* imaging studies have demonstrated a high accumulation level in tumor cells[69].

GA was also used as the targeting moiety to target ASGPRs by Guo *et al*[70]. A pH sensitive nanocarrier system consisting of GA modified alginate/doxorubicin (DOX) modified alginate is prepared and tested on the model of HCC. The release profile of DOX from NPs is prolonged compared to the half-life of free agent and responds to the pH of endosomes. The nanosystem could successfully decrease tumor growth in mice without any mortality.

Varshosaz *et al*[71] also developed galactosylated nanostructured lipid carriers (NLC) for the targeted delivery of 5-FU in HCC. They conjugated lactobionic acid to stearyl amine by chemical reaction. The targeted NLCs of 5-FU contained lecithin, glyceryl monostearate, oleic acid, or Labrafac as the oil phase and was dispersed in an aqueous phase containing Tween 80 or Solutol HS15 as the surfactants. NLCs were prepared by an emulsification-solvent diffusion method. The galactosylated NLCs of 5-FU were cytotoxic at the concentration of half dose of free 5-FU on $HepG_2$ cell line and seemed promising in reducing 5-FU dose in HCC.

Galactosylated chitosan-polycaprolactone (Gal-CH-PCL) NPs loaded with curcumin are another type of the NPs targeted to ASGPRs. NPs have a controlled release and their PCL content is found to be a key factor for the release mechanism. NPs are efficiently uptaken by HepG2 cells *in vitro* and the curcumin loaded into NPs has a 6-fold higher cytotoxic effect than free curcumin. This issue suggests improved bioavailability by Gal-CH-PCL NPs[72].

Another reported example of the targeted nanoparticulate delivery systems is a lactosylated lipid called dioleylphosphatidylethanolamine (Lac-DOPE), which is utilized as the targeting ligand on the modified liposome of methoxy pegylated distearoeylphosphatidylethanolamine (mPEG-DSPE) loaded with DOX which targets ASGPR of hepatocytes in HCC. The Lac-L-DOX nanoliposomes show a higher uptake by HepG2 hepatocellular cells and an enhanced cytotoxicity rate compared with the non-targeted liposomal DOX in conjunction with the longer circulation time due to PEG modification. The *in vivo* studies have also demonstrated increased tumor growth inhibition in nude mice compared to both free and non-targeted liposomal drugs[73].

Galactosylated chitosan (GC) NPs are synthesized encapsulating 5-FU and tested *in vivo* and *in vitro* to

HCC cells. Studies have demonstrated higher apoptosis induction through p53 pathway by GC/5-FU and the test group has no such 5-FU related side-effects as liver injury or bone marrow suppression. These NPs also show a sustained release mechanism for 5-FU, which carrier could be promising for 5-FU delivery to hepatic cells without the usual immunosuppressive effects of this chemotherapeutic agent[74,75]. Another form of these NPs is prepared by cross-linking the GC using norcantharidin as the active pharmaceutical ingredient, whose *in vivo* antitumor activity is better than either the free norcantharidin or norcantharidin attached to CS NPs, but without galactose residue in mice bearing H22 liver tumors[76].

Lactose is another sugar used for targeting ASGPRs. In a study, lactose modified PEGylated poly (amido amine) (PAMAM) dendrimer that is turned into a pH sensitive system by poly (methacryloyl sulfadimethoxine) (LA-PEG-b-PSD-PAMAM) and DOX is loaded in PAMAM. The drug release is significantly higher at pH 6.5 compared to pH 7.4 in PBS. The modified dendrimers have a specific cellular uptake by hepatoma cells at pH 6.5 and *in vivo* studies show a higher tumor growth inhibition rate by the modified dendrimers[77].

Asialofetuin (AF) is a glycoprotein that possesses three asparagine-linked triantennary complex carbohydrate chains with terminal N-acetylgalactosamine residues. This protein has high affinity to ASGPR on hepatocytes and enters the cells through this receptor[78,79]. Thus, AF has been used as a ligand to deliver drugs to hepatocytes and a competitive inhibitor to ASGPR[80,81]. AF-appended liposomes have widespread use as a hepatocyte-selective gene transfer carrier[82-84].

Epirubicin (EPI) loaded chitosan-poly(lactide-co-glicolide) (PLGA) NPs were designed by Nasr *et al*[85] to target hepatocytes using asialofetuin and the NPs were tested on HepG2 cell line and HCC induced mouse model. Also, in an attempt for decreasing cardiotoxicity, these delivery systems were co-administered with tocotrienols. The developed NPs reduced cell proliferation and tumor angiogenesis and also, when co-administered with tocotrienols, further enhanced apoptosis and decreased VEGF level dose dependently. The cardiotoxicity assessment demonstrated that EPI-NPs decreased the level of TNF-alfa induced inflammation, nitric oxide (NO), lipid peroxidation product of oxidative stress, and restored superoxide desmutase levels and also reduced glutathione levels in the heart. All these effects were enhanced when co-administered with tocotrienols.

The ASGPRs are not only used for the targeted delivery of therapeutic agents but also for siRNA based drug delivery. For example, NPs of galactose mediated trimethyl chitosan cystein (GTC) are developed for the oral delivery of VEGF-siRNA and Survivin shRNA-expression pDNA (iSUR-pDNA). Co-administration of a pDNA and siRNA allows for both prompt and long-lasting silencing effects on tumor growth genes. The NPs with moderate galactose density could effectively enter the tumor tissue both *in vitro* and *in vivo* and result in Survivin and VEGF gene silencing and, hence, decreased cell growth and angiogenesis and increased induction of apoptosis. Co-delivery of these two RNAs has a synergistic effect on halting tumor growth compared to single gene delivery[86].

Low-density lipoprotein receptors

HCC is frequently associated with paraneoplastic hypercholesterolemia. In familial hypercholesterolemia, the genetic mutation of low-density lipoprotein (LDL) receptor gene has been recognized as a pathogenesis of the disease[87].

In the study conducted by Chang *et al*[88], hematoporphyrin was used as an LDL target and photo-sensitizing agent in the treatment of HCC. Hematoporphyrin modified bovine serum albumin NPs were loaded with DOX. The designed NPs were evaluated *in vitro* on HepG2 cells and *in vivo* on mice inoculated by HCC. In both cases, efficacy was enhanced according to photodynamic toxicity session and eradiation time.

Cholestrol can also target LDL receptors. Therefore, a nanocomplex consisting of soybean phospholipids and cholesterol conjugated siRNA (Chol-siRNA) for Pokemon gene silencing mediated by reconstituted HDL (rHDL) for targeting HepG2 liver cancer cells was prepared. Pokemone protein is held responsible for oncogenesis in liver cells *in vivo*. NPs have a sustained release kinetic and highly efficient and specific delivery to HepG2 cell line. The *in vitro* studies have shown significant cell growth inhibition and reduction of Pokemon and Bcl-2 proteins (responsible for the inhibition of cell apoptosis) in the treated cells, whereas *in vivo* studies have demonstrated high uptake of carriers by tumor cells of HCC bearing nude mice upon *iv* administration as well as significant cell growth inhibition. Hereby rHDL is suggested as a superior liver cell delivery vector, which facilitates the specific transfection with siRNA[89].

Another reported delivery system which can target LDL receptors is the sterol containing solid lipid nanoparticles (SLNs) that is used for quercetin delivery, a potential chemotherapeutic drug. Low solubility of quercetin seriously limits its clinical use. Therefore, SLNs are designed for enhancing its cellular penetration using cholesterol analogues, *i.e.*, sterols which make bilayers fluent for targeting HCC cells. Three sterol types including cholesterol, stigmasterol, and stigmastanol are used for the preparation of quercetin SLNs by emulsification solvent evaporation method. The IC_{50} of quercetin in cholesterol containing SLNs is about six and twice less than the free drug and phytosterol containing SLNs, respectively, and it causes more accumulation of the drug in HepG2 cells[90].

Ganglioside GM1 cell surface ligand

The branched pentasaccharide chain of ganglioside GM1 is a prominent cell surface ligand, for example, for cholera toxin or tumor growth-regulatory homodimeric galectins[91]. Zhao *et al*[92] used heat-liable enterotoxin subunit B (LTB) as a ganglioside GM1 binding ligand for the targeted treatment of HCC. NPs of the mixture of LTB and bovine serum albumin are prepared and their internalization to hepatocellular cancer cell line SMMC-7721 is tested. 5-FU is loaded in these NPs and cytotoxicity is proved to be much higher than that of the untargeted NPs.

EGFR receptors

Hyperthermia is almost always used along with other forms of cancer therapy, such as radiation therapy and chemotherapy. Hyperthermia may make some cancer cells more sensitive to radiation or harm other cancer cells that cannot be damaged by radiation. There are 2 very different types of hyperthermia: local hyperthermia or thermal ablation in which very high temperatures are used for destroying a small area of cells, such as a tumor. The other way is regional hyperthermia or whole-body hyperthermia in which the temperature of a part of the body (or even the whole body) is raised to a few degrees higher than normal. This type of hyperthermia helps other cancer treatments such as radiation, immunotherapy, or chemotherapy work properly. Local hyperthermia is most commonly done using high-energy radio waves and, consequently, is named RFA to treat tumors up to about 2 inches (5 cm) across. This method is used for the patients in whom surgery is not possible to remove the tumor or for those who have recurrent tumors. It can also be added to other treatments like surgery, radiation therapy, chemotherapy, hepatic arterial infusion therapy, alcohol ablation, or chemoembolization. In this technique, a thin, needle-like probe is put into the tumor for about 10 to 30 min under the guidance of ultrasound, MRI, or CT scans. The high-frequency current produced in the tip of the probe creates heat between 122 °F-212 °F, which destroys the cells within the affected tumor area[93]. In this regard, Raoof *et al*[94] produced some gold NPs loaded with anticancer drug gemcitabin and tested them on the xenograft model of HCC. They used EGFR for the targeted delivery of gemcitabin. As mentioned before, this receptor is expressed on some HCC cell lines such as Hep3B and is specifically targeted by cetuximab as a monoclonal antibody. Using radiofrequency (RF), it induces a non-invasive hyperthermia in targeted cells, but not in normal cells, which results in reduced growth and induction of apoptosis. This study suggests that an Au NP-gemcitabin system could be as effective as the conventional dosage of gemcitabine, but with the almost 275 times less dosage.

VEGF, a sub-family of growth factors, is a signal protein produced by the cells that stimulate vasculogenesis and angiogenesis. When VEGF is over-expressed, it can contribute to the disease. Solid cancers cannot grow beyond a limited size without an adequate blood supply; cancers that can express VEGF are able to grow and metastasize. The influence of siRNA-VEGF on endothelial cell proliferation, apoptosis, and tube formation was analyzed *in vitro* by Raskopf *et al*[28]. Their results showed that two days after transfection, the VEGF expression was inhibited 70% in Hepa129 and 48% in SVEC4-10 cell lines. *In vitro* endothelial cell proliferation and tube formation were reduced by 23% and 38%, respectively. Reduced pAKT in hepatoma cells interfered in VEGF signaling. Intraperitoneal application of siRNA-VEGF inhibited the tumor growth by 83% or 63% in orthotopic tumors within 14 d. VEGF protein was reduced in both models by 29% and 44%. Microvessel density dropped to 34% for the tumors from *ex vivo* transfected cells and 39% for systemic treated tumors.

Human VEGF (hVEGF) siRNA was labeled with ^{131}I using the Bolton-Hunter method and conjugated to a type of SPIOs named SilenceMag. Nude mice with HCC tumors were injected subcutaneously with ^{131}I-hVEGF siRNA/SilenceMag and were then exposed to an external magnetic field (EMF). External application of an EMF attracted and retained more ^{131}I-hVEGF siRNA/SilenceMag in HCC tumors as shown by MRI and biodistribution studies. The tumors treated with ^{131}I-hVEGF siRNA/SilenceMag grew nearly 50% slower in the presence of EMF than those without EMF and the control. Immunohistochemical assay confirmed that the tumor targeted by ^{131}I-hVEGF siRNA/SilenceMag guided by an EMF had a lower VEGF protein level than the one without EMF exposure and the control. The synergic therapy of ^{131}I-hVEGF siRNA/SilenceMag might be a promising future treatment option against HCC with the dual functional properties of tumor therapy and imaging[95].

Specific surface antigens targeted by monoclonal antibodies

One of the most successful therapeutic strategies for solid tumors and hematologic malignancies is based on treatment with the monoclonal antibodies of cancer in the last 20 years. For cancer cell surface antigen discovery, a combination of serological techniques with hybridoma technology leads to a series of landmark clinical trials that paves the way for new generation antibodies and subsequent clinical success. Therapeutic monoclonal antibodies target specific antigens found on the cell surface, such as transmembrane receptors or extracellular growth factors. In some cases, monoclonal antibodies are conjugated to radio-isotopes or toxins to allow the specific delivery of these cytotoxic agents to the intended cancer cell target. One of these therapeutic monoclonal antibodies is Sorafenib (Nexavar), which targets VEGFR, PDGFR, KIT, and RAF antigens and is FDA approved for HCC.

Another antibody with specificity for liver tumor cells is SM5-1 which is a humanized mouse antibody. NPs of PLGA are prepared, conjugated with SM5-1, and loaded with 5-fluorouracil. They are then tested *in vitro* and *in vivo* on subcutaneous and liver tumor HCC-LM3-fLuc cells. In both occasions, the targeted NPs have better efficacy in terms of inhibiting tumor cell growth[96].

Another reported antibody targeted NPs used to specifically target HCC was designed by Wang *et al*[97] who prepared a liposomal NP, mediated by CD44 antibody and loaded with herpes simples virus-truncated thymidine kinase (HSV-ttk), renilla luciferase (Rluc), and red fluorescent protein (RFP) in an attempt to evaluate targeting efficacy by non-invasive molecular imaging. HepG2 cells were injected into the liver of NOD/SCID mice to model *in situ* liver cancer. Then, the growth status of tumor was monitored.

Retinoic acid receptors

Retinoid analogues have been reported to inhibit the growth of HCC. They strongly affect embryogenesis and carcinogenesis. The biological activity of retinoids is exerted through binding to specific nuclear receptors in the steroid/thyroid hormone family. Two major classes of retinoid receptors, RARs and retinoic X, have been identified, each of which consists of three distinct receptor subtypes: α, β, and γ[98]. Retinoic acid receptor-α, is reported as the dominant receptor in HCC and its mRNA has been shown to be at low levels in the normal liver, but at high levels in HCC[52,99]. Retinoic acid is a derivative of vitamin A with an important role in the regulation of cell proliferation and differentiation[100] and its inhibitory effect on cancer cell growth is well established[101-104]. This receptor has been used to target doxorubicin in HCC by Varshosaz *et al*[105]. They graft retinoic acid to chitosan and synthesize copolymers with different degrees of substitution of retinoic acid on the chitosan. Then, the conjugate of retinoic acid-chitosan is grafted to albumin NPs for the targeted delivery of doxorubicin in HCC. NPs are produced by coacervation method. Cytotoxicity of doxorubicin loaded NPs on HepG2 cells using MTT assay shows that IC_{50} of drug loaded in these NPs is reduced to half and one third compared to the non-targeted NPs and free drug, respectively.

Integrin receptors targeted by Arg-Gly-Asp peptide

The tripeptide Arg-Gly-Asp (RGD) was originally identified as the sequence within fibronectin that mediates cell attachment. This tripeptide has been found in numerous proteins, including integrins, a family of cell-surface proteins, which act as receptors for cell adhesion molecules. RGD adheres to integrin receptors, especially $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are over-expressed on the angiogenic endothelium in diseased tissues and various malignant tumors. RGD sequence is the cell attachment site of a large number of adhesive extracellular matrix (ECM), blood and cell surface proteins, and nearly half of more than 20 known integrins recognize this sequence in their adhesion protein ligands. Integrins are transmembrane receptors which act like bridges for cell-cell and ECM interactions. When integrins are triggered, chemical pathways to the interior are activated. Some of these signals include chemical composition and mechanical status of ECM, which result in a response such as the regulation of cell cycle, cell shape, and/or motility or new receptors are added to the cell membrane. This issue allows rapid and flexible responses to events at the cell surface, for example, signal platelets to initiate an interaction with coagulation factors. Proteins that contain RGD attachment site, together with the integrins that serve as receptors for them, constitute a major recognition system for cell adhesion. When RGD peptides are insolubilized onto a surface, they can promote cell adhesion and inhibit it when presented to cells in solution. There are several types of integrins which may be present on the cell surface. Fibronectin, vitronectin, collagen, and laminin (http://en.wikipedia.org/wiki/Ligands) are some of the common ligands for integrins[106]. The binding of a subset of the integrins which recognizes RGD motif within their ligands mediates both cell-substratum and cell-cell interactions. By cyclizing peptides with the selected sequences around the RGD and by synthesizing RGD mimics, it is possible to design reagents that bind selectively to only one or a few of the RGD-directed integrins. Integrin-mediated cell attachment influences and regulates many functions in various biological systems such as cell migration, growth, differentiation, and apoptosis. Therefore, drug design based on RGD structure may provide new treatments for diseases such as thrombosis, osteoporosis, and cancer[107].

Dual-ligand system may possess a synergistic effect and create a more ideal drug delivery effect. Based on the above factors, Mei *et al*[108] designed a multistage liposome system co-modified by RGD, TAT peptide, and cleavable PEG, which combined the advantages of PEG, specific ligand, and penetrating peptide (TAT). TAT is the basic region of the trans-activating transcriptional activator protein from HIV-1[109], which is able to transport different molecules and even 200 nm nanocarriers across biological barriers to be taken up by various cell lines like HCC[110,111]. The cleavable PEG could increase the stability and circulation time of liposomes during circulation. After the passive extravasation to tumor tissues, RGD specifically recognizes the integrins over-expressed on HepG2 cells of HCC and mediates efficient internalization in the synergistic effect of RGD and TAT. *In vitro* cellular uptake and 3D tumor spheroid penetration studies have demonstrated that the system could not only be selectively and efficiently taken up by the cells' over-expressing integrins, but also penetrate into the tumor cells to reach the depths of the avascular tumor

spheroids. *In vivo* imaging and fluorescent images of tumor section have further demonstrated that this system achieves profoundly improved distribution within tumor tissues.

Studies of Cai *et al*[112] demonstrated that the targeting of RGD-coupled to poly(ethylene glycol)-modified stearic acid-grafted chitosan (PEG-CS-SA) micelles to HCC tumor vasculature is a promising strategy for tumor-targeting treatment. DOX was entrapped in the micelles as a model anticancer drug. Qualitative and quantitative analyses of drug-loaded RGD-PEG-CS-SA micelles indicated significant increase of cellular uptake of DOX in HCC cell line (BEL-7402) that over-expressed integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, but not in human epithelial carcinoma cell line (Hela). The competitive cellular-uptake test showed that the cellular uptake of RGD-modified micelles in BEL-7402 cells was significantly inhibited in the presence of excess free RGD peptides. *In vitro* cytotoxicity tests demonstrated that DOX-loaded RGD-modified micelles could specifically enhance cytotoxicity against BEL-7402 compared to DOX-loaded PEG-CS-SA and free DOX.

Carboxy methyl cellulose-magnetic NPs have been synthesized using epithelial cell adhesion molecule (EpCAM) as a target on HCC cells. The *in vitro* MR imaging shows a higher uptake of the targeted magnetic NPs by cancer cells. DOX is loaded to these NPs and the accumulation of DOX in cancer cells is proved higher than that of free DOX or untargeted NPs. This nanoprobe is suggested to be useful for the delivery of therapeutics and imaging compounds[113].

PEG is grafted to polyethylenimine (PEI), modified by RGD tripeptide and functionalized by super paramagnetic iron oxide NPs (RGD-PEG-g-PEI-SPION) for the targeted delivery of Survivin siRNA to human HCC cell line Bel-7402. RGD conjugated NPs have higher efficacy in silencing Survivin gene in cancer cells compared to the non-targeted carriers. This gene suppression in conjunction with induced cell apoptosis leads to tumor growth inhibition in the mouse model of HCC. The SPION functionalized NPs also allow for the efficacious MIR imaging of targeted cells *in vitro* and *in vivo*, which suggests that this specific carrier might be a potential candidate for imaging and treatment purposes in human HCC[114].

Degradable and biocompatible building blocks of amphiphilic derivatives of poly(benzyl malate) are synthesized for the production of functional NPs bearing biotin molecules for the targeted delivery of an anti-cancer model drug, DOX. These NPs target the biotin receptors over-expressed on the surface of several cancer cells. Some of these biotinylated NPs are grafted to cyclic RGD peptide to produce biotinylated cyclic RGD NPs using the strong and highly specific interactions between biotin and the streptavidin protein. The fluorescent NPs grafted with cyclic RGD had A more efficient uptake by the HepaRG hepatoma cells compared to biotinylated fluorescent NPs. Furthermore, the targeting of HepaRG hepatoma cells with NPs bearing cyclic RGD is a very efficient and suitable targeting agent for liver cells[115]. Figure 3 shows the schematic representation of the biotinylated cyclic RGD NPs loaded with DOX.

Knocking down of integrin subunits slows down the progression of HCC, which is due to the significant retardation of HCC progression, reduced proliferation, and increased tumor cell death accompanied by the reduced activation of the *MET* oncogene as well as expression of its mature form on the cell surface. MET is a receptor tyrosine kinase located at the membrane that is essential for embryonic development and wound healing. Hepatocyte growth factor (HGF) is the only known ligand of the MET receptor. MET is normally expressed by the cells of epithelial origin, while HGF expression is restricted to the cells of mesenchymal origin. Upon HGF stimulation, MET induces several biological responses that collectively give rise to a program known as invasive growth. Transformed proliferating cells from HCC are more sensitive to the knock-down of integrins than normal hepatocytes, which highlights the potential of small interfering RNA-mediated inhibition of integrins as an anti-cancer therapeutic approach. All integrin receptors in hepatocytes are down-regulated using the nanoparticulate delivery of short interfering RNAs targeting $\beta1$ and αv integrin subunits. Short-term (2 wk) integrin knock-down does not cause apparent structural or functional perturbations of normal liver tissue. However, sustained integrin down-regulation for 7 wk alters liver morphology[116].

Folate receptors

Folate is a basic component of cell metabolism, DNA synthesis, and repair, and rapidly dividing cancer cells have an increased requirement for folate to maintain DNA synthesis, an observation supported by the widespread use of antifolates in cancer chemotherapy. Because folate receptors (FR) are over-expressed in tumor cells, folate is frequently conjugated with different nanocarriers like polymeric micelles for targeted drug delivery to improve drug efficacy and safety of antitumor drugs[117,118]. FR is a glycosyl phosphatidinositol-anchored membrane protein which is over-expressed in 90% ovarian carcinomas and many types of other epithelial cancers like HCC. Folate receptors (FRα, FRβ, and FRγ) are cysteine-rich cell-surface glycoproteins that bind folate with high affinity to mediate the cellular uptake of folate[119-123].

The expression levels of FR in normal tissues are much lower than in tumor tissues. FRα are especially expressed at high levels in numerous cancers to meet the folate demand of rapidly dividing cells under low folate conditions. FR is an ideal target for drug delivery thanks to its distinct expression between normal and malignant tissues. Folate dependency of many tumors

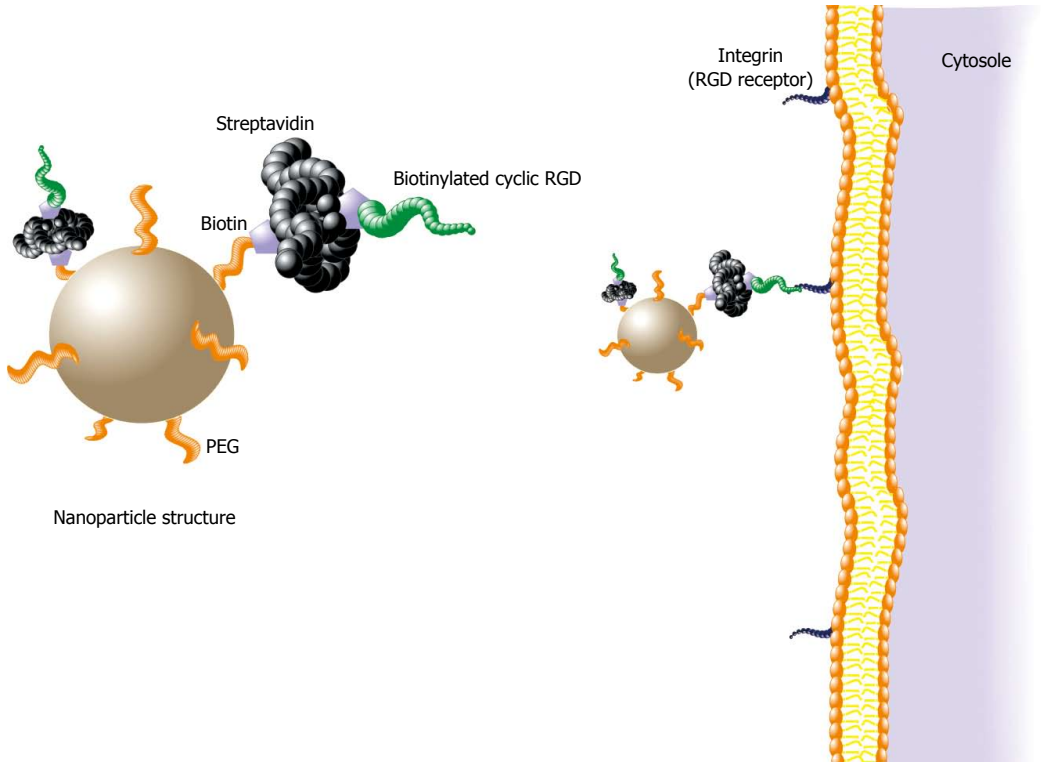

Figure 3 Schematic representation of the biotinylated cyclic Arg-Gly-Asp nanoparticles loaded with doxorubicin. PEG: Polyethylene glycol; RGD: Arg-Gly-Asp.

has been therapeutically and diagnostically exploited by the administration of anti-FRα antibodies, high-affinity antifolates, folate-based imaging agents, and folate-conjugated drugs and toxins. Folate, the natural ligand of FR, has been extensively investigated for chemotherapeutic NP delivery considering its inherent high affinity, small size, and non-toxicity[124-127].

The effect of targeted folate-functionalized polyethyleneglycol-block-poly (e-caprolactone) (PEG-PCL) micelles containing superparamagnetic iron oxide NPs (SPIONs) and sorafenib on the human hepatic carcinoma (HepG2) cells has been studied *in vitro* to observe the feasibility of the surveillance of this targeting therapeutic effect by magnetic resonance imaging. Magnetic resonance imaging using a clinical 1.5 T scanner is performed to detect changes in the signal intensity of cells after incubation with the targeted micelles. The apoptotic rate in the targeted cells is significantly higher than that in the non-targeted cells (P = 0.043). The T2 signal intensity on magnetic resonance imaging of the cells treated with the targeted micelles is significantly decreased with increasing the concentrations of sorafenib in the cell culture medium; but, there is no obvious decrease in signal intensity in the cells treated with the non-targeted micelles. The results of this study show that polymeric micelles functionalized with folate and loaded with SPIONs and sorafenib inhibit proliferation and induce the apoptosis in HepG2 cells *in vitro*. The inhibitory events caused by targeted micelles could be monitored using magnetic resonance[128].

Although the expression of FR on HCC has been proved and its different cell lines like Bel-7402[129-131], Hep3B[132], PLC/PRF/5[133], and HepG2[134,135] have been reported to be FR positive, the last one is controversial and, in some references, it has been used as a FR negative cell line. For example, biodegradable DTX lipid-based nanosuspension (LNS) prepares as the base nanocarrier system. NPs of LNS which are loaded with DTX are either conjugated with folate (fLNS) or coated with PEG (pLNS). These two nanocarriers are tested on the tumor cell line of B16 as an over-expressing folate receptor (FR+) and HepG2 as its under-expressing (FR-). *In vitro* studies have shown no difference between the antitumor effect of tLNS and pLNS in HepG2 cells, whereas *in vivo* studies in B16 bearing mice have demonstrated a higher tumor inhibition level with fLNS compared to pLNS and free agent. Expectedly, the uptake of fLNS is found to be higher after biodistribution studies[136].

Transferrin receptors

Cytotoxicity enhancement of two synthetic derivatives of temozolomide encapsulated in nanostructured lipid carriers (NLC) has been demonstrated for HCC cell lines of HuH-6 and HuH-7[137]. Two types of NPs were designed by Krishna *et al*[138]: one was the NPs of apotransferrin and the second was BSA conjugated to apotransferrin. Both were loaded with DOX and comparative studies were performed. The direct NPs of apotransferrin had a higher uptake by HCC cell's nucleus, even though both were efficiently

internalized by TfR mediated endocytosis. The direct nanodrug showed an improved circulation and kinetic *via* intraperitoneal route and reduced the drug accumulation in the heart *via* iv route; therefore, lower DOX related cardiotoxicity was seen.

Decreased cardiotoxicity of DOX has been also reported by apotransferrin and lactoferrin modified NPs loaded with DOX which are applied in the rat HCC model induced by diethylnitrosamine. Enhancement of drug bioavailability and efficacy in a target-specific mode is also achieved by these NPs[139]. Apo-human serum transferrin coupled with cisplatin could specifically deliver cisplatin to HepG2 cells *in vitro*, minimize the side-effects, and then stimulate apoptosis[140].

In the study by Malarvizhi *et al*[141], DOX was loaded to poly(vinyl alcohol) nano-cores and sorafenib was encapsulated in albumin nanoshell over the nanocore. Sofarenib inhibited the oncogenic signaling and hence a cytostatic effect, while DOX resulted in cytotoxicity. When targeted with transferrin ligand, the cellular uptake and cytotoxicity were enhanced. This core-shell nanoparticle with two relevant medications caused synergistic anti-cancer effects for the treatment of liver cancer.

CONCLUSION AND OUTLOOK

In this article, we highlighted the recent progress of utilizing targeted NPs as delivery system for therapeutic agents and siRNA in the treatment of HCC. According to these studies, targeted NPs have the potential to be used in the delivery of therapeutic agents to the tumor and effective delivery of siRNA to silence the corresponding genetic component involved in this disease. Various materials are used in modifying the physicochemical properties of targeted NPs to increase their stability, loading efficiency, and intracellular delivery, which have led to efficient targeted drug delivery and gene silencing. To prolong blood circulation time, the pegylated version of these nanocarriers can be developed. Surface functionalizing with targeting moiety has been shown to increase tissue accumulation and reduce adverse effects. Great efforts have been made to the coupling of polymer chains to NPs, especially in the context of the so-called bioconjugation for biomedical applications. On the contrary, few works have been reported on the attachment of NPs with different natures for functionalizing agents for efficient co-delivery of siRNA and therapeutic agents. This field certainly needs to be further explored in the upcoming years in order to open very interesting possibilities for the preparation of novel materials through the assembly of functionalized NPs as nonviral inorganic/organic hybrid carriers. At the same time, the pharmacokinetic, pharmacodynamics, safety, and toxicity profiles of these carriers should be considered in preclinical and clinical studies to prove the efficacy of these materials as effective non-viral vectors in gene delivery.

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TOPIC HIGHLIGHT

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Zebrafish as a disease model for studying human hepatocellular carcinoma

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Abstract

Liver cancer is one of the world's most common cancers and the second leading cause of cancer deaths. Hepatocellular carcinoma (HCC), a primary hepatic cancer, accounts for 90%-95% of liver cancer cases. The pathogenesis of HCC consists of a stepwise process of liver damage that extends over decades, due to hepatitis, fatty liver, fibrosis, and cirrhosis before developing fully into HCC. Multiple risk factors are highly correlated with HCC, including infection with the hepatitis B or C viruses, alcohol abuse, aflatoxin exposure, and metabolic diseases. Over the last decade, genetic alterations, which include the regulation of multiple oncogenes or tumor suppressor genes and the activation of tumorigenesis-related pathways, have also been identified as important factors in HCC. Recently, zebrafish have become an important living vertebrate model organism, especially for translational medical research. In studies focusing on the biology of cancer, carcinogen induced tumors in zebrafish were found to have many similarities to human tumors. Several zebrafish models have therefore been developed to provide insight into the pathogenesis of liver cancer and the related drug discovery and toxicology, and to enable the evaluation of novel small-molecule inhibitors. This review will focus on illustrative

examples involving the application of zebrafish models to the study of human liver disease and HCC, through transgenesis, genome editing technology, xenografts, drug discovery, and drug-induced toxic liver injury.

Key words: Cancer model; Hepatocellular carcinoma; Liver disease; Zebrafish; Drug screening

Core tip: Hepatocellular carcinoma is one of the major cancers in the world and involves multiple mechanisms of tumor formation. Recently, the zebrafish has gained acceptance as a platform for developmental biology, drug toxicology, and translational medical research, offering innovative methods for studying disease and cancer formation. In this article, we summarize recent advances in the study of HCC based on the zebrafish as a model system through the use of transgenesis tools, genome editing technology, xenografts, drug hepatotoxicity, and novel drug discovery. Finally, we emphasize how each system works and how the technology was used in this cancer model.

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INTRODUCTION

Liver cancer is one of the world's most common cancers and the second leading cause of cancer deaths, with nearly 745000 deaths recorded in 2012[1]. The incidence of liver cancer is higher among men than women[2]. Hepatocytes are the main cells to constitute 70%-85% of the liver mass and are responsible for the metabolism of carbohydrates, amino acids, lipids, and chemical compounds as well as for the maintenance of the physiological environment[3-5]. Hepatocellular carcinoma (HCC), a primary hepatic cancer, accounts for 90%-95% of liver cancer cases. The pathogenesis of HCC consists of a stepwise process of liver damage that extends over decades, due to hepatitis, fatty liver, fibrosis, and cirrhosis before developing fully into HCC. Chronic liver damage induces genetic alterations of the hepatocytes, leading to cell death, cellular proliferation, dysplasia, and neoplasia. Multiple risk factors are highly correlated with HCC, including infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, aflatoxin exposure[2,6], and metabolic diseases[7] (Figure 1). These factors play critical roles in regulating multiple oncogenes or tumor suppressor genes and activating tumorigenesis-related pathways.

Persistent viral infections are the critical cause of HCC formation. Statistically, 57% of cirrhosis cases and 78% of HCC cases result from HBV and HCV infection[8]. HBV infection causes chromosome instability or insertional mutagenesis[9]. In particular, the HBV X protein (HBx), a small peptide with a molecular mass of approximately 17 kDa, is vital in the pathogenesis of HCC and becomes a prognostic marker of HBV infection and HCC. HBV infection plays an important role in the development of the tumor microenvironment in HCC by regulating the accumulation and activation of both cellular components, such as immune cells and fibroblasts, and non-cellular components of the microenvironment, such as cytokines and growth factors. HBV thus significantly affects the progress of the disease and prognosis[10]. HBx is able to enhance HBV replication, interfere with host gene transcription, interrupt protein degradation, regulate signaling pathways, and deregulate the cell cycle to manipulate cell death[11]. Numerous studies[12,13] have confirmed that the overexpression of HBx causes HCC.

HCV infection is the main risk factor for HCC in developed countries, accounting for approximately one-third to half of all cases. HCV infection leads to activation of Notch and Toll-like receptor pathways in cirrhosis, deregulation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway in early carcinogenesis, and upregulation of DNA replication/repair genes and the cell cycle in the late cancerous stages[14]. HCV proteins such as the core protein and nonstructural protein 5A interact with host cells to regulate processes such as cell signaling, transcriptional modulation, apoptosis, and endoplasmic reticulum stress[15]. A large number of HCV-infected persons develop chronic HCV infection, which can lead to liver fibrosis, cirrhosis, and HCC[16].

Alcohol is a co-carcinogen and is synergistic with the above risk factors for liver cancer. Cirrhosis is observed to have a high correlation with alcohol-associated HCC. Alcohol activates the JAK/STAT and p38 mitogen-activated protein kinase (MAPK) pathways, which responds by producing cytokines, chemokines, and stress. These changes, in turn, affect cell differentiation and growth[17]. Acetaldehyde, a metabolite of alcohol, is even considered to be a carcinogen as it increases oxidative stress and damages DNA[18]. These effects may induce liver fibrosis and cause cirrhosis and HCC development. However, approximately 5%-30% of HCC patients lack any apparent identifiable risk factors for their cancer. Non-alcoholic fatty liver disease (NAFLD) is the hepatic component of metabolic syndromes such as insulin resistance, obesity, hypertension, and hyperlipidemia; it includes both simple steatosis and non-alcoholic steatohepatitis (NASH)[19]. NAFLD/NASH itself becomes a risk factor for HCC, even in the absence of cirrhosis, because insulin resistance and inflammation are involved in HCC carcinogenesis.

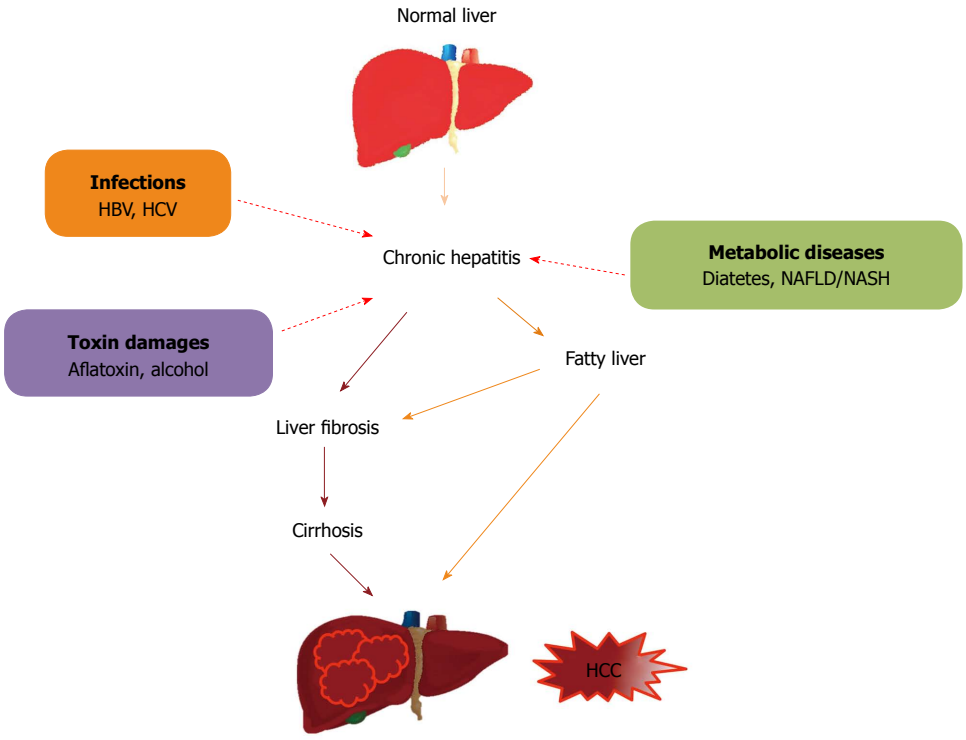

Figure 1 Pathogenesis and risk factors of hepatocellular carcinoma. Hepatocellular carcinoma (HCC) formation results from multiple risk factors, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol, Aflatoxin, and metabolic diseases. These risk factors induce chronic hepatitis, which activates inflammatory pathways. After decades, this inflammatory stress leads to DNA damage and cell cycle dysregulation in hepatocytes, which eventually leads to the development of HCC from chronic disease states, such as liver fibrosis and cirrhosis. Metabolic diseases progress into fatty liver diseases. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Aflatoxin, especially Aflatoxin B1/AFB1, is a genotoxic hepatocarcinogen and a kind of co-carcinogen. AFB1 is metabolized by cytochrome-P450 enzymes to become less harmful metabolites. However, aflatoxin B1-8,9-epoxide (AFBO), the reactive intermediate chemical compound, is a highly reactive genotoxic compound. AFB1 and AFBO both bind to liver cell DNA and form DNA adducts, causing DNA strand breakage, DNA base damage, and oxidative damage[6,20]. AFB1 has been found to accelerate the development of HCC initiated by other risk factors.

The zebrafish has become a model organism exploited in life science fields including embryonic development, toxicity, cancer research, human diseases, and drug screening[21,22]. The genome of the zebrafish is comprised of 25 chromosomes, which contain the full set of genes homologous to other vertebrates. After the current zebrafish genome was fully sequenced, approximately 70% of its orthologous genes were found to be associated with human disease[23,24]. Zebrafish have the following advantages and disadvantages: a short life cycle, low maintenance costs, small space requirements for maintenance, a large number of offspring, immune system deficiencies in early zebrafish embryos, transparency and transgenic lines, lower numbers of cells required for xenotransplantation per animal, availability for high-throughput drug screening, small organs and blood vessels, low body temperature, and a lack of organs such as lungs, among others. Compared to the mouse model, zebrafish are inexpensive and can be easily used to rapidly create a transgenic animal model. Transgenes can be controlled by ubiquitous, inducible, or tissue-specific promoters. Furthermore, transparent zebrafish carry fluorescent proteins that allow the visualization of specific cells in real time. Such capabilities enable investigators to observe and trace specific cells and to produce a spatiotemporal analysis of gene expression. Therefore, the zebrafish is a suitable option for monitoring transgenic tumors from initiation, progression, and metastasis to transplantation. In addition, the zebrafish model can be used for large-scale genetic and high-throughput screening[25,26]. These attributes make the zebrafish a more flexible option among animal models amenable to liver disease studies.

The genes and pathways involved in hepatogenesis and liver cancer are largely conserved between zebrafish and humans[27]. Hepatocytes possess similar functions in zebrafish and mammals and demonstrate similar genesis for shared histopathological characteristics such as steatosis, cholestasis, and neoplasia[28]. Expression of HCV core protein in transgenic zebrafish treated with thioacetamide (TAA) was the first application of zebrafish in HCC studies.

Pathological features observed in this transgenic zebrafish model include steatohepatitis, fibrosis, cirrhosis and HCC. Progression to HCC is reduced to six weeks relative to TAA-treated wild type zebrafish and

becomes a powerful preclinical platform for studying the mechanism of hepatocarcinogenesis in evaluating therapeutic strategies for HCC[29]. Today, genome editing technologies are rapidly advancing. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) systems have been developed to rapidly induce targeted genetic modifications[30]. Such technologies promote the generation of transgenic animal models. This review focuses on the zebrafish model for HCC. We summarize liver development and the anatomy of the zebrafish. Subsequently, expression systems and genome editing technologies are presented to examine the current status of transgenic zebrafish development. Finally, the zebrafish models for liver disease and HCC are introduced for a better understanding of recent findings regarding mechanisms, drug screening, and drug-induced toxic liver injury.

OVERVIEW OF ZEBRAFISH LIVER DEVELOPMENT AND ANATOMY

The liver is a critical organ for vertebrates. Hepatocytes, the cell type which constitutes the majority of the liver, play a major role diverse biological functions, including digestion and metabolism through the regulation of many essential nutrients, the storage of vitamins, the decomposition of red blood cells, the synthesis of plasma proteins such as prothrombin, fibrinogen, and albumins, the production of hormones, and detoxification in mammals[31,32].

The zebrafish is effective as an animal model for studying liver development because of its experimental advantages, as has been demonstrated in numerous publications. However, some characteristics of the zebrafish liver differ from other vertebrates and mammals. For example, hepatocytes are not noticeably organized in cords or lobules, and the typical portal triads are not obvious in the zebrafish liver. The portal veins, hepatic arteries, and large biliary ducts of zebrafish are spread randomly within the hepatic parenchyma and are not grouped into portal tracts as in the mammalian system. Moreover, the hepatocytes are arranged as tubules that surround small bile ducts rather than as bilayered hepatocyte plates as in the mammalian system. The intrahepatic bile ducts are derived from the bile canaliculi. The bile ducts fuse and ultimately form the gallbladder. The bile is collected in the gallbladder *via* large ducts and an extrahepatic biliary system. Moreover, there are no Kupffer cells in the zebrafish liver[33,34]. The liver of zebrafish contains three lobes, one ventral and two lateral, which lie along the intestinal tract. The liver of teleosts is similar to the mammalian liver and plays a central role in metabolic homeostasis, including the processing of carbohydrates, proteins, lipids, and vitamins. In addition, it also plays an important role in detoxification and the synthesis of serum proteins, including albumin, fibrinogen, complement factors, and acute-phase proteins[34].

Similar to the process in the mammalian liver, hepatogenesis occurs in three major phases in zebrafish: hepatoblast specification, budding/differentiation, and hepatic outgrowth, accompanied by morphogenesis[28,35,36]. Cells in the anterior endodermal rod develop into hepatoblasts at 22 h post-fertilization (hpf) through the expression of *hhex* and *prox1* during the hepatoblast specification phase. Hepatoblasts are located on the left side of the anterior gut tube, and the liver bud begins to form between 26 and 28 hpf. Some marker genes, such as ceruloplasmin (cp) and transferrin, are expressed in the liver bud at 32 hpf, with the clear emergence of the liver primordium at 48 hpf during the differentiation phase. The liver bud leaves the intestine at approximately 50 hpf. Due to proliferative acceleration, hepatic outgrowth begins between 60 and 72 hpf and continues until the liver attains its apposite size, and a rapid growth phase of the liver begins at 80-84 hpf[37]. During the end of the outgrowth phase (120 hpf), the liver relocates from the left side to the right side[28].

Signaling molecules and transcription factors are conserved in hepatogenesis between mammals and zebrafish. These pathways also regulate early liver development in zebrafish, though there are some differences. FGF, BMP, and WNT signaling pathways are crucial for hepatogenesis[38,39]. FGF signaling is critical for hepatic specification in zebrafish and mice. Overexpression of a dominant negative FGF-receptor in zebrafish embryos between 18 and 26 hpf decreases the later expression of *hhex*, *prox1*, *gata4*, *gata6,* and cp[40]. A previous study revealed that BMPs are vital for zebrafish hepatic specification: zebrafish mutations such as lost-a-fin or over-expression of a dominant negative BMP receptor led to reduced expression of some hepatic specification genes, such as *hhex* and *prox1*[40]. Induction of *wnt* expression led to a block in liver specification in early somitogenesis and created an enlarged liver after a few hours in zebrafish liver development[41].

Hepatogenesis is a complex process controlled by many transcription factors. The earliest conserved liver-specific transcription factors regulating hepatogenesis, such as *hhex* and *prox1,* are initially expressed and play crucial roles in the zebrafish hepatic bud at 24 hpf[40,42-44]. These hepatic nuclear factors also participate in liver development and differentiation in mammalian hepatogenesis[45]. Some transcription factors, including *sox17*, *foxa1*, *foxa2*, *foxa3*, and *gata* family members are required for both the generation of endoderm and the liver bud[35,46,47]. Recent research indicates that several genes, such as liver-enriched gene 1 (*leg1*), play important roles in the outgrowth stage in zebrafish[48]. Such research has also revealed that hypoxia-inducible transcription factors, such as hif2-alpha, directly regulate the hepatic outgrowth

phase through binding to the promoter region of *leg1* but do not directly regulate the liver specification phase in zebrafish embryos[49].

According to the latest data, there is evidence that epigenetic regulation of gene expression in zebrafish liver development also plays an important role. Histone acetylation (*hdac*) and DNA methylation (*dnmtin*) are two major mechanisms regulating gene expression. An analysis of mutations in *hdac* or *dnmtin* embryos demonstrated that epigenetic regulation controls both hepatic specification and outgrowth phases in zebrafish liver development. For example, *hdac1* mutants develop small livers as a result of hepatic patterning defects[50]. Embryos treated with an *hdac* inhibitor at 24 hpf have also been shown to develop a small liver due to inhibition of *hhex* and *prox1* gene expression. Furthermore, the knockdown of *hdac1* and *hdac3* results in multiple defects in embryos. Aberrant *hdac3* is more specific for liver development as it regulates zebrafish liver growth by suppressing growth differentiation factor 11, a member of the TGF-β family of growth factors[51]. Ubiquitin-like with PHD and RING finger domains 1 (*uhrf1*) plays a role in DNA methylation by recruiting DNA methyltransferase 1 (*dnmt1*) to hemimethylated DNA. The *uhrf1* mutants have defects in zebrafish hepatic outgrowth[52].

CONSTITUTIVE AND INDUCIBLE EXPRESSION SYSTEMS FOR THE DEVELOPMENT OF HCC MODELS IN ZEBRAFISH

Over the past 25 years, the available zebrafish transgenic technology has advanced significantly[53,54]. Transgenesis is an essential technique as in other model organisms. A variety of transgenic expression systems exist for zebrafish, including constitutive and inducible systems. Initial transgenes were plasmid-based with ubiquitous promoters driving the expression of reporter genes and demonstrated that transgenic technology was a viable, reproducible strategy in zebrafish. In recent years, the use of transgenesis in zebrafish has become widespread. Researchers have used a mammalian promoter, promoters from other fish species, and tissue-specific promoters to drive gene expression[55]. In general, the design of most transgenic vectors thus far incorporates a single promoter to control when and where a transgene is expressed. The frequency of the development of germline founders is associated with the method of the introduction of the DNA. Supercoiled[53,56] or linear DNA[54] injection yields 1%-10% germline transgenic founders, while linearized ISce-I[57] meganuclease yield 20%-30%. The rate of transgenesis has recently seen a dramatic increase with the use of transposon-based systems: 30% with Sleeping Beauty[58] and Ac/Ds[59], and 50% with Tol2[60,61]. The Tol2 element is an active transposable element found in *Medaka* genomes, and subsequent production of some cloning vectors has facilitated the use of this element in zebrafish, allowing the generation of many transgenic fish lines[62]. The Tol2 element transposon can be efficiently excised and integrated into the zebrafish genome using coinjection with *Tol2* mRNA and vector plasmid[60]. Cloning vectors from multiple sources, including mini inverted repeat transposons and Tol2 transposase transcription vectors, made use of multisite Gateway cloning vectors[63,64]. In particular, the Tol2-kit was established for the scientific community to allow the use of versatile vectors. Gateway cloning technology is a universal cloning method based on the att site-specific recombination properties of bacteriophage lambda and enables the rapid and highly efficient transfer of DNA sequences into multiple vector systems for protein expression and functional analysis[65]. The online Tol2-kit community (http://tol2kit.genetics.utah.edu/index.php/Main_Page) provides detailed information and has helped to make the Tol2 transposon system a routine genetic engineering tool. Widely useful entry clones were created by combining heat-shock protein 70 (hsp70), CMV/SP6, histone2A-X, β-actin, and upstream activating sequence (UAS) promoters, cytoplasmic, nuclear, membrane-localized fluorescent proteins and Gal4VP16, IRES-driven GFP cassettes, and two Tol2-based destination vectors, one with a Cmlc2/GFP transgenesis marker[64]. One of the most useful GFP transgenic fish lines was derived with a zebrafish liver fatty acid-binding protein (L-FABP) promoter[66,67]. A zebrafish model for hepatocarcinogenesis has been since developed through the expression of oncogenes under the control of the L-FABP promoter. Liver-specific expression of HBx, *src*, and endothelin 1 (*edn1*) established with Tol2 methodology triggered hepatocarcinogenesis in zebrafish[68,69].

While previous studies demonstrated the utility of constitutive expression systems, constitutive expression of oncogenes is often found to lead to gross tumor development which can result in embryonic lethality. Inducible systems can avoid these potentials deficiencies in constitutive systems as the duration and dosage of gene expression can be monitored, thus allowing for the spatiotemporal control of oncogene expression. Inducible systems currently being used include Heat-shock, Cre-loxP, GAL4-UAS, Tet-On, Tet-Off, and Mifepristone systems[70] (Table 1). Heat-shock proteins were originally identified in cells after exposure to environmental stress. Induced jumps in temperature have been used to achieve spatiotemporal control of transgene expression in zebrafish embryos. GFP linked to an hsp70 promoter has been used to establish the pattern of gene expression induced by heat shock. At a normal temperature, GFP expression in transgenic embryos was not detectable. However, single embryos heat-shocked by exposure to 38 ℃ for 30 min exhibited GFP expression in approximately 20%-90% of cells for more than 24 h after heat

Table 1 Advantages and disadvantages of constitutive and inducible expression systems

Expression systems	Advantages	Disadvantages
Constitutive	Well established, commercially available; *in vitro* and *in vivo*, successful methodology for expression of transgene	Expression of oncogenes may cause advanced/highly aggressive tumors and early lethality
Heat-shock	Expression of transgene can be induced on a single cell level	Adverse effects that may arise from the heat shock
Cre-loxP	Well established; commercially available; *in vivo*, successful methodology for expression of transgene	Not all tissue specific promoters are perfectly specific; leaky gene expression; two plasmid system
GAL4/UAS	Well established; *in vivo*, successful methodology for expression of transgene	*In vivo* expression of GAL4 can have side effects, probably related to immune and stress responses; two plasmid system
Mifepristone	Well established; *in vivo*, successful methodology for expression of transgene	Opening and closing of the switch is slow (hours to days); cell permeability of the RU-486 can be restricted
Tet-on/off-inducible	Well established; commercially available; *in vitro* and *in vivo*, successful methodology for expression of transgene	Opening and closing of the switch is slow (hours to days); cell permeability of the doxycycline can be restricted; two plasmid system

treatment in a variety of tissues types[71,72].

Multiple transgenic lines have been derived in zebrafish through the use of tissue-specific expression of Cre recombinase. Initially, a plasmid-based system was developed for detecting Cre expression *in vivo*[73]. A neural progenitor-specific (nestin) promoter was used to drive the expression of an mCherry gene, flanked by loxP sites, and upstream of a promoterless EGFP-fused to zebrafish *kras-V12* oncogene, resulting in the exclusive expression of mCherry. Once this plasmid was exposed to Cre recombinase, the mCherry gene was excised, and the EGFP gene, fused to the oncogene, was controlled by the nestin promoter[74]. The GAL4-UAS system has also been successfully exploited in zebrafish to misexpress genes in a tissue-specific manner. The GAL4-UAS methodology requires two transgenic lines: the activator zebrafish line which expresses the yeast transcriptional activator GAL4 under the control of a specific promoter, and the effector zebrafish line which possesses the transgene of interest fused to the DNA-binding motif (UAS) of GAL4[75,76]. In 1999, an activator line was developed to express GAL4 under the control of the β-actin promoter. In these experiments, the transgene in the effector line encoded a *myc*-tagged protein adjacent to the UAS of GAL4[77]. This report demonstrated that the cross of the effector line with an activator line is necessary for gene expression. This strategy was used to develop an HCC model. In this model, walleye dermal sarcoma virus *rv-cyclin* gene (*orf-A*) fused to the UAS of GAL4 was expressed in the livers of zebrafish when crossed to animals harboring GAL4 under the control of L-FABP promoter[78].

Chemically inducible expression systems (Tet-On, Tet-Off, and Mifepristone) have also been used in zebrafish[79-82]. The Tet-On and Tet-Off systems are binary transgenic systems in which the expression of a transgene is dependent on the activity of an exogenous inducible transcriptional activator. In both the Tet-On and Tet-Off systems, expression of the transcriptional activator can be regulated both reversibly and quantitatively by exposing the transgenic animals to varying concentrations of tetracycline derivatives, such as doxycycline (Dox). The design of the Tet-On and Tet-Off systems allows tissue-specific promoters to drive the expression of the reverse Tet-controlled transcriptional activator (rtTA) and Tet-controlled transcriptional activator (tTA), resulting in tissue-specific expression of the regulated target transgene[55].

Several HCC models have been developed using such technology. Li *et al*[80,83] fused the xiphophorus *xmark* and mouse *myc* oncogenes to the rtTA responsive element, and placed the rtTA transgene was under the control of the 2.0-kb L-FABP promoter. Liu *et al*[81] fused the HBV and HCV oncogenes to the tTA responsive element, and placed the tTA transgene under the control of the 2.8-kb L-FABP promoter. In the mifepristone inducible LexPR system, the LexPR chimeric transactivator was fused to a 2.0-kb L-FABP promoter to produce the driving zebrafish line, and the effector zebrafish line contained EGFP-fused to zebrafish *kras-V12* oncogene under the control of the LexA-binding site. Expression was induced by exposing animals to varying concentrations of mifepristone (RU-486)[81]. In these studies, dose-dependent, Dox, or mifepristone mediated activation of oncogene expression were detected in the liver of the transgenic zebrafish.

GENOME EDITING TECHNOLOGY FOR GENE KNOCKOUT AND LOSS OF FUNCTION IN ZEBRAFISH

Over the past two decades, genetic engineers have made great strides in developing a reliable technique to examine genotypes. Gene knockdown using small interfering RNAs and microRNAs restore the function of dysfunctional genes, but the main disadvantages are off-target interactions and the temporary nature of inactivation achieved through these methods. Today, ZFNs, TALENs, and CRISPR/Cas have become well-established genome editing tools for customizing genomes in human, animal, and plant cells[83-85]. The characteristics and gene editing capabilities in

Table 2 Characteristics of three genome editing systems

Nucleases	ZFN	TALEN	CRISPR/Cas
DNA binding domain	Multiple zinc finger peptides	Transcription-activator like effectors	CRISPR-derived RNA/Single-guide RNA
Endonuclease	Fok1	Fok1	Cas9
Binding specificity of each repeat	3 bp	2 bp	1 bp
Target site length	18 to 36 bp	30 to 40 bp	23 bp
Off-target	High probability	Low probability	Variable
Libraries generation	No	Feasible, depend on technology	Yes, cloning 20 bp, oligos targeting each gene into a plasmid

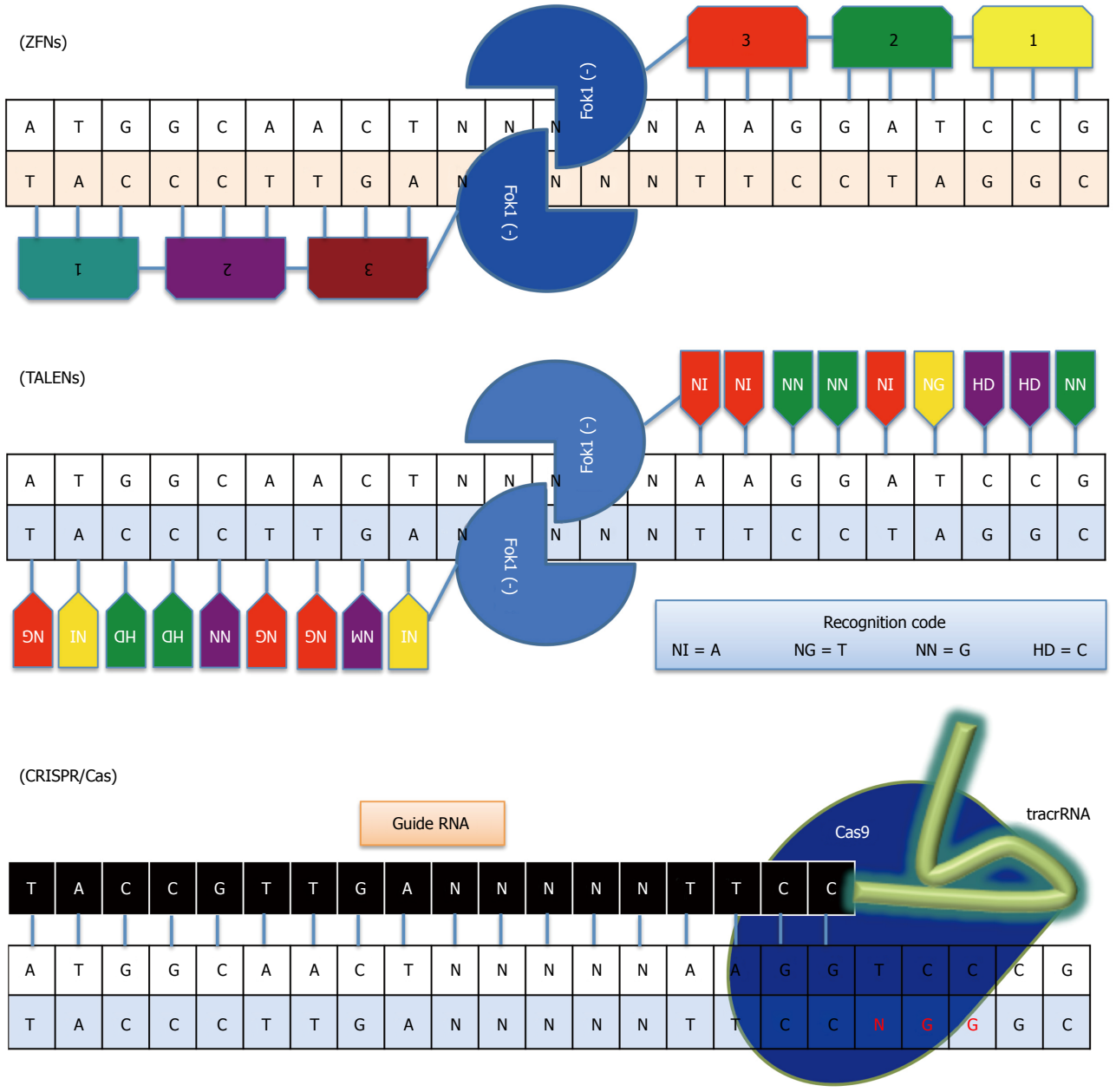

Figure 2 Schematic representation of programmable engineered nucleases of ZFNs, TALENs and CRISPR/Cas.

complex genomes of ZFNs, TALENs, and CRISPR/Cas systems are summarized in Table 2 and Figure 2. RNA-programmable DNA nucleases have been adapted as a precise genetic scissors for correcting and editing genetic defects[86]. Site-specific nucleases induce DNA double strand breaks (DSBs) that stimulate non-homology end joining (NHEJ) and homology-directed repair (HDR) for targeted genomic loci[2].

As one of the numerous DNA-binding motifs in eukaryotic genomes with the ability to recognize

any sequence[87,88], ZFNs are being widely applied to anything in biological research, from the design of animal models to human gene therapies[89]. A ZFN is composed of two domains: a site-specific DNA-binding domain, which is derived from a zinc finger containing transcription factor, and a bacterial Fok1 restriction enzyme endonuclease domain. The zinc finger protein recognizes a 3-bp sequence of DNA on the major groove, with its tandem repeats potentially attaching to a stretch of nucleotides between 9 and 18 bp long[90].

To perform site-specific cleavage of DNA, two ZFN monomers are necessary for the process; one monomer recognizes the binding site on the forward strand while the other recognizes it on the reverse strand. The ZFN binding on both strands enables higher specificity targeting and dimerization of Fok1 in an adequate space[91], so that the pair of Fok1 nuclease domains can cleave the DNA generating a DSB. Cells then utilize either NHEJ or HDR to repair DSBs. The manner in which NHEJ introduces frameshifts into the coding region to knock out a gene, achieved for example through nonsense-mediated mRNA transcript disintegration, is not especially efficient. HDR, however, is used to generate a specific mutation by means of a repair template containing the desired mutation-paired oligonucleotide[92].

Zinc finger proteins with diverse binding specificities are designed using several methods. Modular assembly, which involves a preselected library comprising zinc finger domains for the recognition of 64 nucleotide triplets, is one way of generating customized zinc finger domains[93]. To identify the perfect combination, oligomerized pool engineering utilizes a zinc finger array, and through bacterial-based selection, identifies proteins that bind efficiently to the target site[94]. Other strategies also apply zinc finger modular assembly based on context-dependent DNA to produce ZFNs with endonuclease (endogenous) activities[95]. Although ZNFs offer convenience and are widely utilized, they still possess a high off-target effect[96], which may be improved by developing a heterodimer composed of ZFNs with different Fok1 domains to cleave target DNA[97-99].

TALENs contain a DNA-binding domain and a Fok1 catalytic domain, just like ZFNs, for genomic engineering. A DNA-binding domain is constructed with an N-terminal segment, a central repeat domain, and a half repeat. The central repeat domain is comprised of several monomers that are called transcription activator-like effectors (TALEs). TALEs are effector proteins that are secreted from the bacteria of the *Xanthomonas* genus. They were first found in plant cells, enhancing their susceptibility to pathogens[100]. TALEs are tandem repeats of a 34-amino-acid domain[101], and positions 12 and 13 are known as repeat-variable di-residue (RVD) domains used to determine the specificity of the TALEs. There are four RVD domains, NN, NI, HD, and NG, for the recognition of guanine, adenine, cytosine, and thymidine, respectively[102]. TALEs function as eukaryotic transcription factors *via* DNA binding to activate target gene expression. Fok1 is located in the C-terminal segment and generates a DSB in a spacer sequence. TALENs therefore can be used for targeted gene disruption[103]. It is challenging to construct TALE repeats because each TALE repeat unit has high similarity. Specific methods, such as the restriction enzyme and ligation method[104], Golden Gate cloning[105], and fast ligation-based automated solid-phase high-throughput system[106] have all been designed for the rapid assembly of specific TALENs, so that custom-designed TALENs are in fact a realistic possibility for genetic engineering.

CRISPR/Cas is a prokaryotic defense system against invasion of foreign DNA, utilizing an RNA-guided DNA cleavage system. Small fragments (protospacers) of foreign DNA are inserted at repeat sequences in their own genomes to form CRISPR[107]. The type Ⅱ system consists of a trans-activating crRNA (tracrRNA) in addition to the primary CRISPR RNA transcript (pre-crRNA) transcribed from the protospacers, which is subsequently processed into short crRNAs[108]. To achieve direct sequence-specific DNA recognition and cleavage, CRISPR-associated protein 9 (Cas9) must be complexed with both the crRNA and the tracrRNA, with the crRNA providing the sequence required for target recognition through Cas9. The essential targeting component (5'-NGG-3' protospacer adjacent motif sequence) is located upstream of the crRNA, which is recognized through the Cas9. Through this mechanism, CRISPR/Cas systems cleave the target DNA sequence of 23 bp. Compared to ZFNs and TALENs, the generation of a CRISPR/Cas target specific endonuclease is much easier with the methods of cloning and transcription[109]. Recently, a CRISPR/Cas9 construct was established for tissue-specific gene disruption in zebrafish, and this vector system may become a unique tool to spatially control targeted somatic mutations, gene knockout and loss of function studies in zebrafish[110].

HCC AND LIVER DISEASE MODELS IN TRANSGENIC ZEBRAFISH

The most studied oncogene associated with the development of HCC is the HBx antigen from HBV. HBx has been shown to induce HCC in mice and enhance colony formation in HCC cell lines[111-113]. Transgenic mouse models indicate that HCV is directly pathogenic and oncogenic[114,115]. AFB1 is one of the most prominent carcinogens associated with HCC[116], and it is known to induce formation of DNA adducts and *p53* mutations in liver cell lines[117]. Mutational inactivation of *p53* has been described as one of the key molecular mechanisms involved in the pathogenesis of HCC[118]. AFB1 is synergistic with other factors as AFB1 treatment

induced significantly more liver tumors in HBx and HCV transgenic mice than in wild-type mice[114,115].

Models for liver disease and HCC have been generated in zebrafish through the tissue specific expression of such oncogenes regulated by the L-FABP promoter. In zebrafish, HBx overexpression causes hepatic fat accumulation and liver degeneration in a wild-type background[119]. Tumorigenesis however requires inactivation of the *p53* tumor suppressor pathway, either through mutation of the gene itself or aberrant expression of a negative regulator, such as murine double minute 2 (*mdm2*) protein. Overexpression of *mdm2* alone in the zebrafish liver leads to growth retardation and a fragile liver[120]. Another oncogene affecting the p53 pathways is *gankyrin.* This protein binds ubiquitin protein ligase *mdm2* which promotes *p53* degradation. The inhibition of p53 function through any of these mechanisms prevents the activation of *p53*-dependent apoptotic genes, which leads to cell survival, genomic instability, and oncogenic transformation[121]. Overexpression of *gankyrin* was found to induce hepatic steatosis and regulated miR-16, miR-27b, miR-122, and miR-126. The protein has also been shown to be involved in lipid metabolism[122].

UHRF1 is an important regulator of DNA methylation that is highly expressed in many cancers. *UHRF1* overexpression destabilizes and delocalizes *dnmt1,* causing DNA hypomethylation, *p53*-mediated senescence, and hepatocarcinogenesis in zebrafish[123]. Cyclins are involved in tumor formation and cell death. *rv-cyclin* may also play a role in walleye dermal sarcoma tumor regression by inducing apoptosis[124,125]. Liver-specific expression of walleye dermal sarcoma virus *rv-cyclin* (*orf-A*) in zebrafish protects the fish liver from damage with treatment of 7,12-Dimethylbenz[a]anthracene and delays the onset of malignancy[78].

Edn1 has been identified as a gene that is significantly up-regulated in HBx-induced HCC in the mouse model[126]. Liver-specific induced expression of *edn1* caused steatosis, bile duct dilation, hyperplasia, and HCC in zebrafish[68]. Expression of the transcription factor Yin Yang 1 (*YY1*) was also significantly up-regulated by HBV in a concentration-dependent manner[127]. A previous study has demonstrated through chromatin immunoprecipitation that HBx interacts with YY1[128]. CCAAT/enhancer-binding protein alpha which controls differentiation of hepatocytes was found to be a direct target down-regulated by YY1[129]. Overexpression of *YY1* promoted zebrafish liver steatosis and lipotoxicity by inhibiting C/EBP homologous protein 10 expression[130].

Excessive food intake and increased weight gain to the point of obesity is one of the causes of steatosis. Activation of cannabinoid receptor 1 (CB1R) is a molecular mechanism underlying the regulation of food intake, weight gain, and obesity in mammals. Tet-Off conditional expression of the zebrafish *CB1R* ortholog gene promoted hepatic lipid accumulation and lipotoxicity through the induction of *srebp-1c* expression in zebrafish[131]. In vertebrates, apoptosis is a fundamental part of normal embryonic development and participates in sculpting organs and regulating cell populations. *zfBLP1* and *zfMcl-1a* are functionally similar to members of the *Bcl-2* family, which inhibit apoptosis. Overexpression of *zfBLP1* or *zfMcl-1a* in zebrafish larval liver induced hyperplasia[132].

Combined treatment of zebrafish with HBx and AFB1 induced hepatitis, steatosis, and liver hyperplasia during the early stages of hepatocarcinogenesis[133]. HBx and *src* overexpression induced HCC in *p53* mutant zebrafish and revealed a role for *src* in HCC progression[69]. TAA enhanced the development of steatohepatitis, cirrhosis, and HCC induced by the expression of the HCV core protein in transgenic zebrafish[29]. *In vitro*, the HCV core protein has been shown to directly activate the RAS-RAF-MEK-ERK pathway[134]. In human HCC, Ras proto-oncogenes are activated in as many as 50% of all HCC cases, which leads to activation of downstream signaling pathways including RAF-MEK-ERK and PI3K-AKT-mTOR. Approximately 7% of HCCs carry activating mutations in the *K-RAS* oncogene, which is higher than the percentage of cases carrying *H-RAS* and *N-RAS* mutations. A high level of *kras-V12* expression induced through constitutive or inducible mechanisms initiated liver tumorigenesis in zebrafish[81,135]. The co-expression of HBx and the HCV core protein trigger intrahepatic cholangiocarcinoma in transgenic zebrafish[80]. However, transgenic zebrafish over-expressing HBx or HCV individually do not develop HCC.

Recently, expression of *kras-GV12* and *xmrk*, the homolog of mammalian epidermal growth factor receptor oncogene, in zebrafish with Tet-On conditional methodology has been reported as an outstanding model for revealing new therapeutic targets involved in oncogene-regulated hepatocarcinogenesis[79,136]. RNA sequencing analysis of an *xmrk* transgenic HCC model revealed a potential role for immune responses in HCC progression and regression. This model may provide molecular insight into the targeted inhibition and significance of immune response in tumor regression[137].

The liver is one of the most important organs for the study of autophagy[138]. In fact, liver tumors are one of the main phenotypes in knockout mice of autophagy-related genes[139]. In zebrafish, the EGFP-Lc3 transgenic line crossed with the *xmrk* transgenic line yielded animals susceptible to HCC and thus, demonstrated that autophagy plays an important role in HCC development[140]. Cross-species analyses demonstrated that Tet-On conditional expression of *myc* in a zebrafish model paralleled findings in *myc* mouse models for HCC. Elevated *myc* expression in zebrafish caused liver hyperplasia, adenoma, and HCC. *Myc*-induced liver tumors in zebrafish also possessed molecular signatures that were similar to those from

mouse and human HCC. This zebrafish model thus revealed a conserved role for *myc* in promoting hepatocarcinogenesis in all vertebrate species[82]. RNA expression profiling of liver tumors from the three different zebrafish models, *xmrk*, *kras-G12V,* and *myc,* showed however relatively little overlap in significantly deregulated genes and biological pathways. However, these three transgenic tumor signatures were found to be significantly correlated with advanced or late stage human HCC[141].

In human HCC, deregulation of MYC is frequently detected and correlated with poor prognosis. Two differentially expressed *MYC* orthologs exist in the zebrafish genome: *myca* and *mycb*. Overexpression of *myca* and *mycb* in the liver using a mifepristone-inducible system demonstrated that both *myc* genes were oncogenic. *myca* overexpression accelerated tumor progression and reduced apoptosis in *p53* mutant zebrafish. Malignant hepatocytes were dependent on sustained *myca* expression; withdrawal of the mifepristone inducer resulted in a rapid regression of HCC, with liver tumor regression occurring even in a *p53* mutant background[142].

RhoA is a member of the RHO small GTPase family, which is highly homologous to the RAS. These proteins are also involved in the regulation of cell cycle dynamics, and are key molecules for cell growth and tissue development of the switch. Expression levels and the overall activity of RhoA has been found to be elevated in HCC[143]. Tet-On conditional expression of *kras-G12V*, *rhoA*, constitutively active *rhoA-G14V,* dominant-negative *rhoA-T19N,* or *kras-G12V* plus one of the three *rhoA* genes, was also examined in zebrafish. Overexpression of *kras-G12V* during early development led to liver enlargement and hepatocyte proliferation. The increase in liver size was augmented by the dominant-negative *rhoA-T19N,* but abrogated by the constitutively active *rhoA-G14V*. This study revealed the existence of signaling crosstalk between *kras-V12* and *rhoA* in regulating liver overgrowth and hepatocarcinogenesis[136]. Based on these results, the zebrafish emerges as a model system for elucidating the mechanisms of hepatocarcinogenesis and for screening drugs to inhibit the oncogenic effects of specific genes (Table 3).

POTENTIAL APPLICATIONS OF XENOGRAFTS AND ZEBRAFISH HCC MODELS IN DRUG DISCOVERY

The United States Food and Drug Administration approves only a few new chemical entities for clinical usage each year because the investigation of new drugs is a lengthy and costly process. Drug-discovery generally proceeds first through *in vitro* assays, where cell proliferation, cytotoxicity, marker expression, motility, activation of specific signaling pathways, and changes in morphology are examined in response to treatment with small molecules[144], and second through *in vivo* screening where endpoints such as extended life span can be evaluated. The zebrafish has the advantage of combining both processes in a single model. It is a high-throughput and *in vivo* model simultaneously; therefore, the zebrafish might improve the success rate in the later stages of preclinical drug development while reducing the cost and the time necessary for the screening process[70].

The trend of using zebrafish embryos in screening for anti-cancer drugs continues to rise. The use of computational drug design and screening of zebrafish embryos has successfully uncovered a novel lead compound that displays selective inhibitory effects on CDK2 activity, cancer cell proliferation, and tumor progression *in vivo*[145].

Zebrafish/tumor xenograft models have been used to study angiogenesis, invasion, and metastasis. One advantage of zebrafish is that the embryos are transparent, allowing the observation of labeled tumor cells and the evaluation of response to candidate molecules in a high-throughput format *in vivo*[146]. In order to achieve maximum transparency, zebrafish embryos are incubated in an egg medium with 0.3% phenylthiourea to prevent the formation of pigments. (In the mouse system, the spatial resolution is limited *in vivo* due to normal opacification of the skin and subdermal structures). Tumor cells labeled with CM-DiI, a lipophilic fluorescent tracking dye, are injected into the perivitelline space or yolk of embryos at 48 hpf and are followed thereafter. *fli1*:gfp transgenic embryos and the whole-mount akaline phosphatase vessel staining assay allows for rapid and relatively easy investigation of tumor angiogenesis, cell dissemination, invasion, metastasis, and anti-vascular endothelial growth factor (VEGF) drugs for cancer therapy[68,147]. Transgenic zebrafish (*vegfr2*:grcfp) where GFP expression is restricted to blood vessels have been used to screen a compound library for antiangiogenic compounds. SU4312 and AG1478, two known anti-angiogenic compounds, were used as positive controls in the screen. Two new compounds with no previously described antiangiogenic activity, indirubin-3'-monoxime (IRO) and EM011 (9-bromonoscapine), were also identified[148,149]. Embryos of the transgenic *flk*:gfp zebrafish were also used in screening the compound library. One lead compound, rosuvastatin, was identified which could inhibit the growth of the zebrafish intersegmental vessels[150]. The zebrafish tumor xenograft model represents a new tool for investigating the neovascularization process and is exploitable for drug discovery as well as gene targeting in tumor angiogenesis.

In zebrafish HCC models, mifepristone-induced *kras-V12* transgenic larvae treated with MEK1/2 inhibitor PD98059 resulted in the inhibition of hyperplastic liver growth in 49% of cases. Inhibition of PI3K-AKT-mTOR signaling by LY294002 or rapamycin restored the normal liver phenotype in 57% and

Table 3 Zebrafish animal models of liver disease and hepatocellular carcinoma

Transgene name	Expression system	Liver pathology	Ref.
cnr1 (Zebrafish)	Tet-off-inducible	Steatosis	[132]
edn1 (Zebrafish)	Constitutive	Steatosis, bile duct dilation, hyperplasia and HCC	[69]
gankyrin (Zebrafish)	Constitutive	Atrophy, hypoplasia and steatosis	[123]
HBx (Human)	Constitutive	Hypoplasia and steatosis	[120]
HBx + *AFB1* (Human)	Constitutive	Hepatitis, steatosis and hyperplasia	[134]
HBx + *HCV* (Human)	Tet-off-inducible	Intrahepatic cholangiocarcinoma	[81]
HBx + $p53^{M214}$ (Human)	Constitutive	Chronic inflammation, steatosis, bile duct dilation, dysplasia and HCC	[70]
HBx + *src* (Human/Zebrafish)	Constitutive	Chronic inflammation, steatosis, bile duct dilation, dysplasia and HCC	[70]
HCV (Human)	Constitutive	Steatosis	[29]
HCV + *TAA* (Human)	Constitutive	Steatosis and HCC	[29]
kras-G12V (Zebrafish)	Mifepristone	Hyperplasia and HCC	[82]
kras-G12V (Zebrafish)	Constitutive	Hyperplasia and hepatocellular adenoma	[82]
kras-G12V (Zebrafish)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[137]
kras-G12V + $p53^{M214}$ (Zebrafish)	Constitutive	Hyperplasia and hepatocellular adenoma	[82]
kras-G12V + *RhoA* (Zebrafish)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[137]
kras-G12V + *RhoAG14V* (Zebrafish)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[137]
kras-G12V + *RhoAT19N* (Zebrafish)	Tet-on-inducible	HCC	[137]
Lc3 (Rat)	Constitutive	Investigation of liver autophagy	[141]
mdm2 (Zebrafish)	Constitutive	Atrophy, contraction and hypoplasia	[121]
MYC (Mouse)	Tet-on-inducible	Hyperplasia and hepatocellular adenoma	[83]
myca (Zebrafish)	Mifepristone	Small, typical, hypervascular and ascites of liver tumor	[143]
myca + *p53M214* (Zebrafish)	Mifepristone	Small, typical, hypervascular and ascites of liver tumor	[143]
mycb (Zebrafish)	Mifepristone	Small, typical, hypervascular and ascites of liver tumor	[143]
orf A (Human)	GAL4/UAS	Delayed onset of liver tumor	[79]
src (Zebrafish)	Constitutive	Chronic inflammation, steatosis, bile duct dilation, hyperplasia, dysplasia and HCC	[70]
src + *p53M214* (Zebrafish)	Constitutive	Steatosis, hyperplasia, dysplasia and HCC	[70]
UHRF1 (Human)	Constitutive	Atypical cells, dysplastic foci and HCC	[124]
UHRF1 + *p53M214* (Human)	Constitutive	Atypical cells, dysplastic foci and HCC	[124]
xmrk (Xiphophorus)	Tet-on-inducible	Hyperplasia, hepatocellular adenoma and HCC	[80]
yy1 (Zebrafish)	Constitutive	Steatosis	[131]
zfBLP1 (Zebrafish)	Constitutive	Hyperplasia	[133]
zfMcl-1α (Zebrafish)	Constitutive	Hyperplasia	[133]

HCC: Hepatocellular carcinoma; HBx: Hepatitis B virus X protein; HCV: Hepatitis C virus.

69% of *kras-V12* transgenic larvae, respectively. Results furthermore demonstrated that blocking two pathways in *kras-V12* transgenic larvae resulted in a more significant anti-tumor effect (78%-96%)[81]. Recently, liver tumors were induced in doxycycline regulated *xmrk* transgenic fish with 100% penetration in both juveniles and adults. Overexpression of *xmrk* activated downstream targets of MEK1/2 and STAT5, which led to increased cell proliferation during tumor progression and enhanced apoptosis during tumor regression. Juvenile fish were also exposed to MEK1/2 inhibitor PD98059 or STAT5 inhibitor nicotinohydrazide in combination with doxycycline. After three weeks of treatment, abdomens and livers in 100% of transgenic fish exposed to either inhibitor were reduced relative to untreated transgenics[79]. Transient expression of the HCV core protein under the control of a CMV promoter, human hepatic lipase promoter, and zebrafish L-FABP enhancer in zebrafish embryos was used as a possible model to examine HCV replication and treatment with drugs. The amplified sub-replicon was evidence of high expression of HCV core RNA and protein. This model was used to evaluate efficacy of four HCV clinical drugs: oxymatrine, ribavirin, IFNa-2b, and vitamin B12. Vitamin B12 inhibited HCV core mRNA and protein levels in a dose-dependent manner. Ribavirin and oxymatrine drugs also significantly inhibited replication of the HCV sub-replicon. Such models may provide a novel strategy for studying mechanisms of HCV replication as well as facilitate the discovery of new anti-HCV drugs[151-153].

ZEBRAFISH MODELS FOR STUDYING DRUG-INDUCED TOXIC LIVER INJURY

Drug-induced liver injury (DILI) is a major problem in clinical pharmacology. Here, zebrafish is also promising as an animal model[25]. Zebrafish is a high-throughput *in vivo* model that can be potentially used to predict which therapeutic compounds will cause DILI in humans as well as present new markers and molecular mediators of DILI. One of the most important features of the model is that drug metabolization in zebrafish is mediated through similar pathways utilized in humans[154]. Different methods have been used in order to evaluate and quantify DILI in zebrafish. Although higher vertebrate organisms that are physiologically similar to humans have typically been used to assess DILI, the zebrafish has

similar molecular and cellular processes that accurately simulate human physiology. Therefore, zebrafish provide a significant advantage for research purposes compared to higher vertebrate organisms (*e.g.*, mice and rats). For example, the ability to assess liver damage with visually evaluable phenotypic endpoints enables the transparent larval zebrafish to be used in high-throughput screening[155,156]. In addition, DILI in embryonic or adult zebrafish exhibits histological changes, such as steatosis, apoptosis, and necrosis, that parallel human liver pathologies[157]. TAA has been shown to induce steatohepatitis in zebrafish, which is accompanied by the accumulation of fatty droplets and apoptosis[158]. AFB1 induced hepatitis and steatosis in zebrafish[133]. Zebrafish exposed to ethanol also exhibited histological changes such as steatosis, as found in alcoholic liver disease in humans[159]. Serum biochemical values, such as total bilirubin concentration and serum alanine transaminase (ALT) activity, have been determined in zebrafish[160]. Such values can be therefore used to evaluate liver function in response to drug treatment. ALT activity was found to be increased in zebrafish treated with paracetamol in a dose and time dependent fashion[157]. Furthermore, the circulating concentration of miR-122, a new experimental biomarker for liver toxicity, was increased in fish with paracetamol-induced liver injury[161].

Although many studies clearly illustrate the potential advantages of zebrafish as a model for liver toxicity, a number of challenges still exist. For example, zebrafish are exposed to a drug simply by introducing it into the water[162]. Immersion in the drug enables easy and fast administration, but the amount actually consumed by the fish is a variable even though the concentration is known and equal for all fish[163]. To overcome the problem of absorption, the quantity of the drug taken up by the fish can be determined by using a radio-labeled compound and liquid scintillation counting[154].

Before the zebrafish model can be more broadly applied, translatability of the model to humans must be confirmed. First, tests need to be conducted on established human hepatotoxic and nonhepatotoxic compounds, comparing dose responses between fish and humans. Second, translational biomarkers that bridge the gap between fish and humans must also be developed. Finally, immunological response in zebrafish must be evaluated in order to establish whether DILI develops similarly as in humans. The use of zebrafish as a model for liver injury shows promise and may enable better decision making in the early stages of drug discovery, before a compound is tested in higher mammals.

CONCLUSION

HCC is a primary malignant tumor of the liver. It is a complex disease that is accompanied by an overall poor prognosis. Although numerous oncogenes, tumor suppressor genes, and point mutations have associated with development of the disease over the past several decades, treatment options remain limited. One of the more intriguing approaches to the study of HCC and potential treatments, has been through the development of HCC disease models in zebrafish. Several zebrafish HCC models have been established through expression of various transgenes, including HBx, HCV, *myc*, *kras-G12V*, *rhoA*, *xmrk*, *src*, *edn1*, *myca*, *mycb*, or *UHRF1*. Zebrafish models have also been used for evaluation of DILI and tumor xenotransplantation. Recently, new genome editing technologies, including ZFNs, TALENs, and CRISPR/Cas systems, have been developed to facilitate targeted gene disruption in zebrafish. Together with transgenic technology, several inducible expression systems are also available for zebrafish, which will help to accelerate further development of fish models for HCC. Although establishment of liver disease and HCC models in zebrafish has led to further understanding of the molecular mechanisms and biology of these diseases, zebrafish perhaps more importantly serve as *in vivo* models with high throughput screening capabilities for the discovery of novel therapeutic agents. Novel inhibitors of angiogenesis, IRO and EM011, have been identified through such screening technology. As the utility of zebrafish for the study of HCC becomes more universally accepted, we will perhaps facilitate drug discovery and thus one day advance our treatment and the prognosis of HCC patients.

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TOPIC HIGHLIGHT

2015 Advances in Hepatocellular Carcinoma

Sorafenib-based combined molecule targeting in treatment of hepatocellular carcinoma

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Abstract

Sorafenib is the only and standard systematic chemotherapy drug for treatment of advanced hepatocellular carcinoma (HCC) at the current stage. Although sorafenib showed survival benefits in large randomized phase Ⅲ studies, its clinical benefits remain modest and most often consist of temporary tumor stabilization, indicating that more effective first-line treatment regimens or second-line salvage therapies are required. The molecular pathogenesis of HCC is very complex, involving hyperactivated signal transduction pathways such as RAS/RAF/MEK/ERK and PI3K/AKT/mTOR and aberrant expression of molecules such as receptor tyrosine kinases and histone deacetylases. Simultaneous or sequential abrogation of these critical pathways or the functions of these key molecules involved in angiogenesis, proliferation, and apoptosis may yield major improvements in the management of HCC. In this review, we summarize the emerging sorafenib-based combined molecule targeting for HCC treatment and analyze the rationales of these combinations.

Key words: Angiogenesis; Mammalian target of rapamycin; Extracellular-signal regulated kinase; Endothelial growth factor receptor; Histone deacetylases

Core tip: Cancer is regarded as a heterogeneous disease, with no exception of hepatocellular carcinoma (HCC), which requires combined chemotherapy. HCC is not sensitive to most currently used conventional cytotoxic drugs. The approval of sorafenib, a molecular targeted drug that inhibits RAF kinase and several other angiogenesis-related receptor tyrosine kinases, opens a door for systematic treatment of HCC. The pathogenesis of HCC involves hyperactivation of several

signal pathways and aberrant expression of some key molecules, suggesting combination treatment may yield major improvements in the management of this disease. The emerging sorafenib-based combination treatments are reviewed in the present article.

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INTRODUCTION

Liver cancer is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746000 deaths in 2012 according to the statistics published by World Health Organization[1]. Among the diverse, histologically distinct primary hepatic neoplasms, hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for 83% of all cases[2-4]. The prevalence of HCC is especially severe in China and Japan due to high rates of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in the population[5-8]. Curative treatments including liver transplantation and hepatic resection are only suitable for fewer than 20% of HCC patients because most cases have progressed to an advanced stage with intra- or extra-hepatic metastasis when the disease is diagnosed[9-13]. As a palliative treatment, chemotherapy is a highly needed means for the patients with unresectable or metastatic HCC[14-17].

HCC is generally recognized as a chemo-resistant tumor that is not sensitive to most currently used cytotoxic drugs[18-20]. Recent discoveries in the molecular mechanisms of HCC pathogenesis have created many opportunities for developing targeted therapies[21-24]. Sorafenib, which targets RAF kinase (c-RAF and b-RAF) and several other angiogenesis-related receptor tyrosine kinases (RTKs) including vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR), is the first approved molecular targeted agent that has been applied to systemic chemotherapy in HCC patients with metastatic disease or transcatheter arterial chemoembolization (TACE)-refractory disease[25,26]. Although clinical studies demonstrated some benefits of sorafenib on the time to progression (TTP) and overall survival (OS), its efficacy against HCC remains moderate[25]. The median TTP was demonstrated to be 5.5 mo for sorafenib and 2.8 mo for placebo, and the median OS was 10.7 mo for sorafenib and 7.9 mo for placebo[25]. Results of subsequent clinical trials on single agents including linifanib, brivanib, and sunitinib are disappointing since they are not superior to sorafenib or placebo in terms of efficacy or safety[27]. At this stage, there have not yet been any verified drugs except for sorafenib in systematic treatment of HCC.

Combination of sorafenib and other molecular targeted drugs for treatment of HCC has drawn wide attention in recent years based on the following understanding: (1) HCC is a highly heterogeneous disease in terms of etiology and clinical behavior, which signifies that cancer-driven genes may be varied in tumors of different patients[28]; (2) Cell clones in one tumor are heterogeneous with respect to both morphology and function, which means that the tumor is made up of diverse cell populations that show different drug sensitivity[29,30]. The drug-resistant clones may survive under the evolutionary selection pressure and then drive the cancer progression; and (3) Hepatocarcinogenesis is a stepwise process during which multiple genes are altered[31]. Genetic changes and their biological consequences in cancerous cells may include tumor suppressor genes, oncogenes, reactivation of developmental pathways, growth factors and their receptors, and angiogenesis[32]. The complexity of pathogenesis of HCC suggests that combination of drugs that target multiple key molecules implicated in tumor initiation and progression is a promising strategy to conquer this pressing disease. In this review, we summarizes recent advances of sorafenib-based combined treatments for HCC (Figure 1 and Table 1).

COMBINING SORAFENIB AND ANTIANGIOGENIC AGENTS

HCC is a hypervascular tumor and angiogenesis plays an important role in the progression of the disease[33]. Studies revealed that CD34, a sensitive angiogenesis-related endothelial marker, was not detected in healthy liver and cirrhotic liver, but was obviously expressed in HCC, suggesting that angiogenesis is probably a driving force in HCC development[34,35]. Regarding the molecular mechanisms of angiogenesis, VEGF is the best known angiogenic factor that promotes endothelial cell (EC) proliferation and migration[36-38]. Besides, various VEGF-independent drivers have also been recognized, including multiple interactions among diverse growth factors and receptors involving EC, delta-like ligand 4 (DLL4), angiopoietin (Ang)-Tie, placental growth factor (PIGF), tumoral cells (SDF1/CXCR4), pericytes [platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β)], extracellular matrix (ECM) components (integrins and cadherins), inflammatory cells (tumor-associated macrophages and Tie-2-expressing monocytes), and bone marrow-derived cells[39,40]. The antiangiogenic effect of sorafenib is mainly ascribed to its ability of inhibiting VEGFR-2, PDGFR, and these RTKs-mediated RAF/MEK/ERK pathway[41]. Although sorafenib

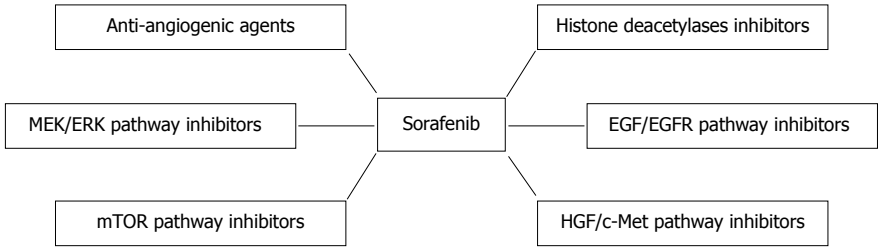

Figure 1 Overview of sorafenib-based combined targeting therapies for hepatocellular carcinoma. MEK: Mammalian target of rapamycin; ERK: Extracellular-signal regulated kinase; EGFR: Endothelial growth factor receptor; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor.

Table 1 Landscape of sorafenib-based combined molecule targeting in treatment of hepatocellular carcinoma

Combined drugs	Treatment modality	Current stage	Outcome	Ref.
Antiangiogenic agents				
Dalantercept	Simultaneous combination with sorafenib	Phase Ⅰb (ongoing)	Awaited	NCT02024087
Axitinib	Sequential treatment after sorafenib failing	Phase Ⅱ (finished)	Warranting further clinical study	[49]
	Sequential treatment after sorafenib failing	Phase Ⅱ (ongoing)	Awaited	EUCTR2011-002029-24-IT
Apatinib	Sequential treatment after sorafenib failing	Phase Ⅲ (ongoing)	Awaited	NCT02329860
MEK/ERK inhibitors				
AZD6244	Simultaneous combination with sorafenib	Phase Ⅱ (terminated)	Unknown	NCT01029418
Refametinib	Simultaneous combination with sorafenib	Phase Ⅱ (finished)	Warranting further clinical study	[64]
mTOR inhibitors				
Sirolimus	Simultaneous combination with sorafenib	Preclinical study	Synergistic effect	[73]
Everolimus	Simultaneous combination with sorafenib	Phase Ⅱ (ongoing)	Awaited	NCT01005199
Temsirolimus	Simultaneous combination with sorafenib	Phase Ⅱ (ongoing)	Awaited	NCT01687673
PI-103	Simultaneous combination with sorafenib	Preclinical study	Synergistic effect	[86]
PKI-587	Simultaneous combination with sorafenib	Preclinical study	Synergistic effect	[87]
HDACs inhibitors				
Panobinostat	Simultaneous combination with sorafenib	Phase Ⅰ (terminated)	Precluding phase Ⅱ studies	NCT00873002
Vorinostat	Simultaneous combination with sorafenib	Phase Ⅰ (suspended)	Unknown	NCT01075113
Resminostat	Simultaneous combination with sorafenib	Phase Ⅱ (finished)	Warranting further clinical study	[100]
	Simultaneous combination with sorafenib	Phase Ⅰ/Ⅱ (ongoing)	Awaited	NCT02400788
MPT0E028	Simultaneous combination with sorafenib	Preclinical study	Synergistic effects	[97]
EGFR inhibitors				
Erlotinib	Simultaneous combination with sorafenib	Phase Ⅲ (finished)	Not recommended	[109]
c-Met inhibitors				
MSC2156119J	Sequential treatment after sorafenib failing	Phase Ⅰ/Ⅱ (ongoing)	Awaited	NCT02115373
Tivantinib	Sequential treatment after sorafenib failing	Phase Ⅲ (ongoing)	Awaited	NCT02029157
	Simultaneous combination with sorafenib	Phase Ⅰ (finished)	Warranting further clinical study	[118]
DE605	Simultaneous combination with sorafenib	Preclinical study	Synergistic effects	[117]

HDAC: Histone deacetylase; EGFR: Endothelial growth factor receptor.

exhibits reliable antiangiogenic effects, the complexity of angiogenesis suggests that it cannot block the formation of tumor microvessels completely[42]. The redundancy of angiogenic mechanisms may contribute to drug resistance through activation of alternative proangiogenic pathways. In this sense, combination of sorafenib and other antiangiogenic agents with different targets may improve the efficacy of sorafenib monotherapy and minimize the arising of drug resistance.

Activin receptor-like kinase-1 (ALK1) is a type Ⅰ, endothelial cell-specific member of the TGF-β superfamily of receptors with high affinity for bone morphogenetic protein-9 (BMP9) and BMP10. Multiple lines of evidence demonstrated that BMP9/BMP10/ALK1 pathway is implicated in blood vessel formation and organization[43,44]. Dalantercept, a soluble form of ALK1 that prevents activation of endogenous ALK1 by BMP9 or BMP10, inhibits maturation of vascular endothelial cells, disrupts vascular development, and displays potent antitumor activity in preclinical models[45,46]. Distinguished with sorafenib, dalantercept targets this alternative angiogenic pathway and blocks common downstream events in the angiogenic process like the later vascular maturation stage. Thus combining dalantercept and sorafenib may achieve enhanced inhibition of tumor angiogenesis. It is worth noting that the side-effects of dalantercept

mainly include lower extremity peripheral edema (grades 1-2) and congestive cardiac failure (grades 1-3), which do not appear to overlap with the toxicity profile of sorafenib[47]. Currently, an open-label, multi-center phase 1b study (NCT02024087) is ongoing to evaluate the safety, tolerability, pharmacokinetics, pharmacodynamics, preliminary activity, and the recommended phase 2 dose of dalantercept and sorafenib when used in combination in advanced HCC. This clinical study will also examine the biomarkers associated with tumor response including BMP9/10 and ALK1 in tumor biopsies and serum.

In parallel with the endeavors exploring the efficacy of simultaneous combination of sorafenib and antiangiogenic agents, studies have also been performed to investigate whether antiangiogenic drugs are available as the second-line therapy to control disease progression when sorafenib treatment fails. Axitinib is a multiple tyrosine kinase inhibitor targeting VEGFR1, VEGFR2, VEGFR3, PDGFR, and c-Kit[48]. It has been approved by the United States Food and Drug Administration for treatment of advanced kidney cancer. Preclinical studies demonstrated that the drug also exhibited potent activity against liver cancer[49]. A phase Ⅱ trial (NCT01334112) of second-line axitinib following prior antiangiogenic therapy (sorafenib or bevacizumab) in advanced HCC showed that axitinib induced partial response in 1 of 26 patients and stable disease in 10 of 26 patients with a 16-wk cutoff, equivalent to a response rate of 3.8% and tumor control rate of 42.3%, which met the primary end point of the study[50]. The common adverse events in the axitinib group were hypertension, fatigue, dysphonia, and hypothyroidism[50]. Overall, axitinib showed encouraging clinical activity in patients pretreated with antiangiogenic agents in this study. Currently, there is also an ongoing multi-center, second-line study (EUCTR2011-002029-24-IT) of axitinib in patients with advanced HCC that progressed with sorafenib. The primary endpoint of the study is to assess the rate of patients without progression in an evaluation period of 4 mo and the results are still awaited.

Another antiangiogenic agent that is currently being evaluated as the second-line option for advanced HCC that acquires resistance to sorafenib is apatinib, which is a tyrosine kinase inhibitor selectively targeting VEGFR2[51]. *In vitro* studies demonstrated that it effectively inhibited proliferation, migration, and tube formation of human umbilical vein endothelial cells as well as blocked the budding of rat aortic ring[52]. *In vivo* studies showed that apatinib was capable of inhibiting the growth of several established human tumor xenografts[52]. A phase Ⅰ study of apatinib showed encouraging antitumor activity and a manageable toxicity profile[53]. An interesting feature of this compound is that it can circumvent the multidrug resistance of cancer cells to certain conventional drugs by inhibiting the functions of multidrug resistance-associated proteins such as ABCB1 and ABCG2[54,55]. This characteristic of apatinib indicates that it might be useful in combining other drugs for cancer treatment. Thus far, a phase Ⅱ trial (NCT01192971) of apatinib in patients with advanced HCC has been completed but the results have not been published. A phase Ⅲ trial (NCT02329860) sponsored by the same company, *i.e.*, Jiangsu HengRui Medicine Co., Ltd. (Jiangsu, China), was started on December 2014, aiming to evaluate the efficacy and safety of apatinib in patients with advanced HCC who have progressed on targeted therapy such as sorafenib and/or chemotherapy.

COMBINING SORAFENIB AND MEK/ERK INHIBITORS

Except for the antiangiogenic effect, sorafenib can directly suppress the proliferation of HCC cells, which is ascribed to its ability of inhibiting RAF kinase and thus blocking the RAF/MEK/ERK signal pathway[56]. It is known that RAS/RAF/MEK/ERK represents a dominant signaling pathway promoting cell proliferation and survival[56,57]. The binding of different growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) to their receptors induces activation of RAS which in turn activates RAF, MEK, and ERK[58]. The activated ERK translocates into the nucleus and then activates transcription factors that regulate the expression of genes involved in cell proliferation and survival[58]. The RAS/RAF/MEK/ERK pathway is in an activated status in the majority of advanced HCC cases due to increased signaling induced by upstream growth factors such as EGF, HGF, and IGF[58,59]. Thus, inhibiting this signal pathway may significantly suppress the growth of HCC, which has been evidenced by many studies thus far. Although sorafenib is capable of interfering the signal transduction of this pathway *via* inhibiting the functions of RAF, its efficiency may be compromised by the complementary and/or feed-back mechanisms, which may partially restore the levels of phosphorylated ERK[60,61]. In order to overcome this issue, studies were performed to assess the efficacy and safety of combining sorafenib and MEK/ERK inhibitors in treatment of HCC. Thus far, some favorable results have been achieved in preclinical and clinical studies.

Huynh *et al*[60] reported that sorafenib significantly inhibited the growth of HCC xenografts but also caused elevation of insulin-like growth factor receptor 1 (IGF-1R) and phospho-c-RAF Ser 338 (activated form of c-RAF). The underlying mechanisms were revealed to be related with inhibition of c-RAF Ser 259 by sorafenib. It was reported that the phosphorylation of c-RAF at Ser259 prevented c-RAF activation, and that the dephosphorylation of c-RAF at Ser259 was an essential part of the c-RAF activation process[62]. It is possible that inhibition of phospho-c-RAF Ser 259 by sorafenib facilitates the phosphorylation of c-Raf at Ser338 which in turn promotes the phosphorylation

of MEK and ERK. This finding warrants evaluation of the efficacy of combining sorafenib and MEK inhibitors in HCC treatment. As expected, inhibition of MEK by a small molecular compound AZD6244 (also named ARRY-142886) obviously enhanced the antitumor effect of sorafenib in both orthotopic and ectopic models of HCC[60]. A phase Ⅱ trial (NCT01029418) was started in December 2009 to evaluate the efficacy of combination of AZD6244 and sorafenib in treatment of advanced HCC. Unfortunately, this trial was terminated without any definite conclusions due to funding issue in April 2015. Further studies are needed to verify the strategy of combining sorafenib and AZD6244 in treatment of HCC.

Another feed-back model involved in the RAF/MEK/ERK pathway also demonstrated the rationale of simultaneous inhibition of RAF and MEK in treatment of HCC. It has been described that the activated ERK could inhibit RAF, which is a negative feed-back regulation of RAF/MEK/ERK signal transduction[63]. Inhibition of MEK/ERK signaling by MEK inhibitors relieves ERK-dependent feedback inhibition of RAF and then compensatorily induces MEK phosphorylation[63,64]. Thus, MEK inhibitors combining sorafenib that inhibits RAF may block the feed-back loop and efficiently inhibit the RAF/MEK/ERK signal transduction. Interestingly, this model of action for MEK feed-back regulation is true for refametinib (BAY 86-9766)[61], an orally available small molecule that binds to an allosteric region adjacent to the ATP-binding pocket of MEK and inhibits both MEK 1 and MEK 2 with high potency and selectivity. In preclinical studies, refametinib exhibited potent antiproliferative activity in HCC cell lines and was strongly synergistic with sorafenib in suppressing the growth of HCC xenografts[61]. At the signaling pathway level, the combination of refametinib and sorafenib led to inhibition of the upregulatory feedback loop toward MEK phosphorylation observed after refametinib monotreatment[61]. In a completed single-arm phase Ⅱ trial (NCT01204177), disease control rate (DCR), TTP, and OS were higher compared with previous sorafenib monotherapy studies (44.8% *vs* 35.3% for DCR; 4.1 mo *vs* 2.8 mo for TTP; 9.7 mo *vs* 6.5 mo for OS), especially in relation to Asian patients[65]. With regard to the toxicity, both refametinib and sorafenib were tolerated; however, most patients required dose modifications, mainly due to frequent grade 3 adverse events. Overall, combining refametinib and sorafenib provides a potential option for patients with unresectable HCC, which warrants a phase Ⅲ trial for further evaluation.

COMBINING SORAFENIB AND AGENTS INHIBITING PI3K/AKT/MTOR SIGNAL PATHWAY

Similar to the RAF/MEK/ERK pathway, PI3K/AKT/mTOR is also a major intracellular signaling pathway that regulates multiple cellular functions, including cell growth and proliferation, motility, survival, apoptosis, autophagy, and angiogenesis[66-68]. It has been described that the PI3K/AKT/mTOR signaling pathway plays a pivotal role in HCC and is activated in 30%-50% of HCC cases[69]. Many inhibitors targeting this pathway are currently being evaluated for HCC treatment in preclinical and clinical studies. Studies demonstrated that the RAF/MEK/ERK pathway could be activated as a consequence of mTOR inhibition, which might attenuate the antitumor effects of mTOR inhibitors[70,71]. On the other hand, inhibition of RAF/MEK/ERK signaling by sorafenib could induce increased mTOR phosphorylation, especially in sorafenib-resistant HCC cell lines[71,72]. The fact that blockage of RAF/MEK/ERK or PI3K/AKT/mTOR, separately, can result in activation of the other pathway underscores the potential of a combined therapeutic approach with agents targeting these two pathways. Another favorable factor of this combination strategy is that some mTOR inhibitors such as sirolimus and everolimus can be used as immunosuppressants to prevent organ rejection response after liver transplantation[73]. Thus, combination of mTOR inhibitors and sorafenib confers both anticancer and immunosuppressive properties, which might be suitable for treatment of recurrent HCC after liver transplantation.

Consistent with the theoretical assumption, addition of mTOR inhibitors such as sirolimus, everolimus, and temsirolimus to sorafenib augments antitumor effects in HCC preclinical studies *in vitro* and *in vivo*[74-76]. The issue of this combination strategy is that patients with advanced HCC showed lower tolerance to mTOR inhibitor or sorafenib compared to monotherapy. In a phase Ⅰ study of the combination of everolimus and standard dose of sorafenib (400 mg twice daily) in advanced HCC, the maximum-tolerated dose (MTD) of everolimus was recommended to be 2.5 mg daily which is one third or fourth of the MTD established in everolimus monotherapy for advanced HCC[77]. Everolimus escalation beyond 5 mg daily in the combination settings was showed to be accompanied by dose limited toxicities such as grade 3 aspartate aminotransferase (AST) elevation, hyperbilirubinemia, and grade 3/4 thrombocytopenia. However, based on the effective dose of everolimus, a phase Ⅱ trial (NCT01005199) is currently ongoing to assess the efficacy of sorafenib (400 mg twice daily) plus everolimus (5 mg daily) in treating patients with localized, unresectable, or metastatic liver cancer. Similar with everolimus, temsirolimus combined with sorafenib also results in reduced MTD of both drugs. A phase Ⅰ dose-finding trial demonstrated that the MTD and recommended phase 2 dose of the combination strategy are temsirolimus 10 mg weekly plus sorafenib 200 mg twice daily[78], which are both lower than each single-agent MTD as well as the combination MTD identified in melanoma patients without hepatic dysfunction, which is temsirolimus 25 mg weekly

plus sorafenib 400 mg in the mornings and 200 mg in the evenings[79]. A phase Ⅱ trial (NCT01687673) is currently ongoing to evaluate the efficacy of temsirolimus (10 mg weekly) plus sorafenib (200 mg twice daily) in treatment of advanced HCC.

The efficacy of combination therapy with mTOR inhibitors and sorafenib for recurrent HCC after liver transplantation was initially described in several case reports, implying the usefulness of this combination regimen[80,81]. Due to concerns of the toxicity of mTOR inhibitors and sorafenib when used in combination, two clinical retrospective analyses were performed to assess the efficacy and safety of this combination modality for recurrent HCC after liver transplantation[82,83]. A single-center retrospective study analyzed the data of 7 patients receiving everolimus and sorafenib treatment and showed that the regimen is challenging regarding side effects and requires close patient monitoring to adapt everolimus dosage to sorafenib exposure and toxicity[83]. The other clinical study analyzed the data of 31 patient who were concomitantly administered everolimus or sirolimus plus sorafenib and confirmed the effectiveness of this combination strategy described in previous case reports[82]. A significant but manageable toxicity profile of mTOR inhibitor plus sorafenib was also observed in this study, which cautions that a careful assessment of potential vascular or hemorrhagic complications should be performed for all patients. The study suggests that combined treatment with mTOR inhibitor and sorafenib might be a therapeutic option for post-transplant HCC recurrence not amenable to curative treatments, which warrants further randomized and controlled clinical studies in the future.

The above mentioned mTOR inhibitors including sirolimus, everolimus, and temsirolimus specifically target mTORC1 (mechanistic target of rapamycin complex 1), which is one of the two components of mTOR[84]. Because they do not directly inhibit the other component of mTOR, mTORC2, that is also vital to tumor maintenance and progression, the action mode of these drugs does not fully exploit the antitumor potential of mTOR targeting in cancer[85]. Additionally, mTORC1 can also negatively regulate PI3K, implicating potential feedback activation of PI3K by sirolimus, everolimus, or temsirolimus[86]. An agent that is able to inhibit both mTORC1 and mTORC2, and also block upstream of mTOR, will result in a stronger inhibition of the PI3K/AKT/mTOR pathway. PI-103 and PKI-587 are representatives of this class of drugs, which showed potent inhibitory effects on various human cancers including HCC in pre-clinical studies[87,88]. Gedaly *et al*[71,88] demonstrated that PI-103 plus sorafenib or PKI-587 plus sorafenib could synergistically inhibit EGF-stimulated HCC cells proliferation compared with monotherapy. Further studies revealed that activation of PI3K/AKT/mTOR by EGF was blocked by PI-103 or PKI-587 which, however, stimulated MEK and ERK phosphorylation; activation of RAS/RAF/MEK/ERK by EGF was suppressed by sorafenib which, on the other hand, induced AKT and mTOR phosphorylation[71,88]. Either PI-103 or PKI-587 combining with sorafenib could inhibit all the tested kinases in the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways, which accounts for the superior effects of combination regimen to single drug in suppressing the proliferation of HCC cells. These studies reinforce the rationale of simultaneous inhibition of HCC pivotal pathways Ras/Raf/MEK/ERK and PI3K/AKT/mTOR.

COMBINING SORAFENIB AND HISTONE DEACETYLASES INHIBITORS

Histone deacetylases (HDACs), along with histone acetyltransferases, reciprocally regulate the acetylation status of the positively charged NH2-terminal histone tails of nucleosomes[89]. Acetylation of histones by HDACs reduces the affinity of histones for DNA, resulting in an open DNA structure that facilitates gene expression[89,90]. HDAC inhibitors are currently considered to be among the most promising anticancer agents in drug development and some of them such as panobinostat and vorinostat have been approved for marketing in treatment of multiple myeloma and cutaneous T cell lymphoma[91,92]. It has been demonstrated that overexpression of HDACs is associated with poor prognosis and survival rates of HCC patients[93]. The efficacy and safety of HDAC inhibitors including resminostat and belinostat in treatment of advanced HCC have been validated in phase Ⅱ clinical trials[94,95], indicating the potential value of HDAC inhibitors for HCC therapy.

The strategy of combining HDAC inhibitors and sorafenib for enhanced antitumor efficacy has been tested in HCC. Preclinical studies showed that addition of HDAC inhibitors including panobinostat, vorinostat, and MPT0E028 to sorafenib achieved additive or synergistic effects against HCC both *in vitro* and *in vivo*, providing the rationale for clinical studies with this combination regimen[96-98]. Panobinostat, a non-selective HDAC inhibitor (pan-HDAC inhibitor), was once sporadically reported to enhance the antitumor effect of sorafenib in patients with advanced HCC[99,100]. However, the subsequent phase Ⅰ trial (NCT00873002) was terminated due to dose limiting toxicity, which precluded phase Ⅱ study of this combination in treatment of advanced HCC. Vorinostat, an inhibitor of HDACs 1 and 3, is currently under phase Ⅰ evaluation (NCT01075113) when combination of sorafenib in the setting of HCC, which is now suspended for interim analysis of dose limiting toxicity. Among HDAC inhibitors that are evaluated as combined drugs with sorafenib, resminostat that inhibits the activities of HDACs 1, 2, and 3 was testified to be a promising option. A recent multi-center, phase Ⅱ Shelter study (NCT00943449) carried out in 8 German and 6 Italian centers validated the efficacy and safety of

resminostat plus sorafenib for advanced HCC patients. Results demonstrated that combination of resminostat and sorafenib yielded a striking 70% progression free survival (PFS) rate at 12 wk[101]. Moreover, the treatment improved the OS of patients from 3 mo to 8 mo. Encouraged by these findings, a phase Ⅰ/Ⅱ study (NCT02400788) was implemented in Japan and South Korea in March 2015 to assess the availability of resminostat plus sorafenib in Asian patients with advanced HCC. While much work remains to be done, the currently achieved results open a door to better treatment prospects for this devastating disease.

The mechanisms underlying the synergistic effects of combination of HDAC inhibitors and sorafenib against HCC have been revealed from the following several aspects. First, sorafenib inhibits the RAF/MEK/ERK pathway and causes increased expression of Wnt-pathway regulator CDH1, a pivotal player of hepatocarcinogenesis known to be implicated in growth arrest[102]. Because HDACs can directly suppress CDH1 expression, combination of HDAC inhibitors and sorafenib results in a further rise of CDH1 expression, which might partially explain the observed potent antitumor effects of the combined therapy[96]. Second, HDAC inhibitors such as MPT0E028 is capable of activating ERK and its downstream molecules *via* induction of FGFR3-mediated signaling, suggesting that HDAC inhibitors may render HCC cells more dependent on ERK signaling[98]. Abrogation of RAF/MEK/ERK signaling by sorafenib may sensitize tumor cells to HDAC inhibitor-induced cell apoptosis[98]. Third, HDAC inhibitors can upregulate signal transduction pathways related to angiogenesis by modulating the expression of growth factors like VEGF or of downstream kinases like mitogen activated protein kinases (MAPKs), which are also the targets of sorafenib[103,104]. The dual blockade of tumor cell proliferation and tumor angiogenesis could thus represent a molecule basis for the synergistic effects of the combination therapy.

COMBINING SORAFENIB AND AGENTS TARGETING EGFR OR C-MET

The EGFR pathway was demonstrated to be implicated in the pathogenesis of several cancers including HCC[58]. As an orally active inhibitor of EGFR tyrosine kinase, erlotinib has been approved to treat patients with advanced non-small cell lung and pancreatic cancers. In terms of HCC, two single-arm phase Ⅱ trials showed that erlotinib exhibits modest antitumor activity but promising OS benefit[105,106]. It was reported that EGFR activation may interfere with HCC response to sorafenib, indicating that EGFR inhibition may enhance the antitumor effects of sorafenib[107]. Although preclinical studies showed no improvement of sorafenib efficacy upon combination with erlotinib in an orthotopic rat model of HCC[108], the combination showed promising antitumor efficacy (including HCC) in a phase Ⅰ trial[109]. Encouraged by the results of this clinical trial, phase Ⅱ trials were skipped and a phase Ⅲ study (NCT0901901) was directly performed to compare the efficacy and safety of first-line sorafenib/erlotinib with sorafenib/placebo in patients with advanced HCC. Recently published results of this study demonstrated that median OS and median TTP were similar in the sorafenib plus erlotinib and sorafenib plus placebo groups[110]. With regard to the safety profile, the rates of treatment-emergent serious adverse events (AEs) and drug-related serious AEs were comparable in the two arms. The lack of synergistic or additive effect of the combination of erlotinib and sorafenib suggests that EGFR signaling may not be pivotal in advanced HCC. Thus, application of EGFR inhibitors plus sorafenib is not recommended to treat advanced HCC.

Like EGFR, the mesenchymal-epithelial transition factor (c-Met) is also a typical member of RTKs. HGF and des-γ-carboxy prothrombin (DCP) are revealed to be high-affinity ligands for c-Met[10,111,112]. Aberrant activation of the c-Met signaling pathway plays a critical role in cancer progression and metastasis by promoting cell proliferation, survival, and motility, suggesting that c-Met is a promising target for cancer therapy[113]. Overexpression of c-Met alone has also been demonstrated to be sufficient for developing HCC in c-Met-transgenic mice[114]. In addition, c-Met overexpression is observed in 20%-66% of human HCC samples and is closely related with patients' prognosis[10]. It is worth noting that c-Met expression in HCC cells was upregulated after sorafenib treatment, which might be related with tumor response to the drug[115]. Studies by the current authors and other researchers showed that inhibition of c-Met kinase led to an obvious antitumor effect in HCC, indicating that targeting c-Met is also a promising strategy for HCC treatment[26,59]. Indeed, a randomized, placebo-controlled phase Ⅱ trial showed that tivantinib, a selective inhibitor of c-Met, almost doubled median TTP and median OS in patients with high c-Met-expressing HCC who failed sorafenib treatment[116]. A confirmatory phase Ⅲ, randomized, placebo-controlled trial (NCT02029157) is currently ongoing to evaluate the efficacy and safety of tivantinib in HCC patients who had high c-Met expression in their tumors and developed progressive disease under sorafenib therapy. Besides tivantinib, MSC2156119J, another c-Met inhibitor that shows potent inhibitory effects on the proliferation and metastasis of HCC cells *in vivo*[117], is currently under phase Ⅰ/Ⅱ study (NCT02115373) to assess its efficacy, safety, and pharmacokinetics in subjects with c-Met positive advanced HCC who have failed sorafenib treatment. Besides a second-line treatment option, c-Met inhibitors have also been tested for its efficacy upon combination with sorafenib. A recent report shows that sorafenib and DE605, a novel c-Met inhibitor, synergistically suppress the growth of HCC both *in vitro* and *in*

vivo[118]. A phase I study of tivantinib in combination with sorafenib in patients with advanced solid tumors including HCC showed that the combination of tivantinib (360 mg, bid) plus sorafenib (240 mg, bid) was well tolerated and might have therapeutic potential in this setting[119]. Further studies are warranted to evaluate the efficacy and safety of this combination in the future. The above studies suggested that c-Met inhibitors may be combined as one of the options in sorafenib-based combination therapy.

CONCLUSION

Sorafenib remains the standard care for first-line treatment of advanced HCC. Unfortunately, the benefits of sorafenib may not be sustained, which requires alternative effective treatment regimens. The molecular pathogenesis of HCC is very complex, involving different pathways and molecular aberrations such as RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, VEGF, c-Met, and HDACs, which warrants multiple-targeted therapeutic approaches. Simultaneous or sequential abrogation of these critical pathways or the functions of key molecules may yield major improvements in the management of HCC.

Several issues revealed in the past studies require attention when the combination regimen is translated from bench to bedside. First, the failure of combination of sorafenib and erlotinib in the phase Ⅲ trial may be ascribed to the absence of phase Ⅱ trials that assess the efficacy and safety of this combination in advanced HCC although promising results of phase Ⅱ trials of erlotinib monotherapy are available. Future phase Ⅲ trials should select agents and regimens with proven favorable safety profiles in HCC-specific phase I trials and documented efficacy based on well-designed and preferably randomized phase Ⅱ trials. Second, clinical evidence suggests that combination regimen may be more often associated with dose-limiting effects, whereas single drugs can be used at maximal dosing level without causing intolerable toxic effects. Concerns over the toxicity of amplified drug toxicity in combination treatment trials have been the current bottleneck to translating positive preclinical experiments into clinical trials in HCC. This has been the case with the combination of sorafenib and mTOR inhibitors. Thus, combination of drugs that have none or less overlapped toxicity profiles and drug-drug interactions may minimize the risk of amplified adverse effects. Third, achievement of favorable therapeutic effects with combination regimens may also require patient screening to identify a responsive subset, which is critical for successful development of molecule targeted drugs and helpful for the subsequent individualized treatment. Identification of biomarkers that can predict or monitor tumor response to the treatment can facilitate this process and should be highlighted in the future. The heterogeneity of HCC determines the diversity of treatments. The emerging various kinds of tested sorafenib-based combination regimens may meet this requirement and help better manage this pressing disease.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Liver transplantation for hepatocellular carcinoma - factors influencing outcome and disease-free survival

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Abstract

Hepatocellular carcinoma is one of the leading causes of cancer-related death worldwide. Liver transplantation can be a curative treatment in selected patients. However, there are several factors that influence disease-free survival after transplantation. This review addresses the pre-, intra- and postoperative factors that influence the risk of tumor recurrence after liver transplantation.

Key words: Hepatocellular carcinoma; Survival; Risk factor; Diagnostics; Recurrence; Liver transplantation

Core tip: Hepatocellular carcinoma is one of the leading causes of cancer-related death worldwide. Liver transplantation can be a curative treatment in selected patients. This review addresses the pre-, intra-, and postoperative factors that influence disease-free survival and the risk of tumor recurrence after liver transplantation. Furthermore, novel diagnostic methods are presented and discussed.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide[1,2]. Most HCC co-occurs with liver cirrhosis, and the etiology of liver cirrhosis leading to HCC differs among global regions, *e.g.*, hepatitis C virus infection in North

Table 1 Overview of the selection criteria for liver transplantation

Name	Criteria	Ref.
Milan	1 tumor < 5 cm in diameter or ≤ 3 tumor nodules, each ≤ 3 cm in diameter No extrahepatic manifestation No vascular invasion	Mazzaferro *et al*[7]
Up-to-seven criteria ("new Milan")	Seven as the sum of the size of the largest tumor (in cm) and the number of tumors	Mazzaferro *et al*[166]
Kyoto	≤ 10 tumors, all ≤ 5 cm in diameter PIVKA-II > 400 mAU/mL	Takada *et al*[167]
UCSF	Solitary tumor ≤ 6.5 cm or ≤ 3 nodules with largest lesion ≤ 4.5 cm and total tumor diameter ≤ 8 cm No gross vascular invasion	Yao *et al*[9]
Shanghai Fudan	Solitary tumor ≤ 9 cm in diameter or ≤ 3 lesions with the largest ≤ 5 cm and total tumor diameter ≤ 9 cm	Fan *et al*[168]
Hangzhou	Total tumor diameter ≤ 8 cm or Total tumor diameter more than 8 cm with histopathological grade I or Ⅱ and preoperative α-fetoprotein ≤ 400 ng/mL	Zheng *et al*[169] Lee *et al*[170]
Asan	Largest tumor diameter ≤ 5 cm Hepatocellular carcinoma number ≤ 6 No gross vascular invasion	

America, Europe, and Japan and hepatitis B virus infection in China, South Korea, and Taiwan[1].

Depending on hepatic function, liver transplantation (LT) is a curative option for selected HCC patients. However, the recurrence rate after LT is reported to be between 5% and 23%[3-6].

This review addresses the issue of HCC recurrence after LT and is subdivided into three sections: preoperative, intraoperative, and postoperative factors.

PREOPERATIVE FACTORS

Size and number of HCC nodules

In 1996, Mazzaferro *et al*[7] introduced the Milan criteria (MC), which showed a survival benefit in HCC patients with one tumor nodule < 5 cm diameter or three tumor nodules with a maximum diameter < 3 cm for each nodule. The MC remain the gold standard for decision-making in LT settings for HCC patients.

The MC have been challenged by different authors and work-groups aiming at an expansion of the criteria (see Table 1). However, there is only a consensus that "modest expansion of the criteria should consider the dynamics of the waiting list and whether a worse prognosis could be tolerated, if there is no prejudice for patients without HCC"[4].

What we do know is the so-called "Metroticket paradigm": the larger the tumor burden, the lower the post-transplant expected survival. However, reliance on tumor size and number of nodules is not the most ideal approach because biological parameters, such as the response to local-ablative therapy or tumor grading, are not included.

In practice, there are two important pipelines in liver allocation: center-based allocation, meaning that a graft is offered to a center that chooses the ideal recipient for a donor organ, and MELD-based allocation, which involves a waiting list where patients with a high MELD score receive an organ sooner than patients with a low MELD score. This is important in HCC patients because most have good hepatic function and subsequently a low MELD score. These patients have the chance of requesting an "exceptional" MELD score when fulfilling certain criteria. This phenomenon brings us back to the MC: in most countries around the world, the MC is the deciding tool for an exceptional MELD request (see Table 2).

However, there is increasing evidence that a decision on organ allocation based only on radiological findings does not reflect the reality of tumor biology. A large single-center study of over 800 patients undergoing LT because of HCC revealed that in addition to tumor size, other clinicopathological parameters are helpful and necessary to identify patients with a lower risk of tumor recurrence[8]. Varona *et al*[5] showed that the French prognostic model, including pre-transplant α-fetoprotein (AFP), tumor size, and number of nodules, was better at detecting patients with HCC recurrence after LT than the MC or up-to-7 criteria. Yao *et al*[9] showed that expanded tumor size had no negative effect on patient survival or tumor recurrence. In conclusion, the development of the MC was an important step towards improved outcome after LT in HCC, but these criteria are likely too narrow, and further adaptations are necessary.

AFP

The biomarker AFP, which is encoded in humans by the AFP gene located on chromosome 4[10-12], is a frequently used serum parameter for the detection of HCC. Unfortunately, the sensitivity of AFP is limited because non-tumor liver diseases are also associated with high serum AFP levels[13,14], and AFP levels are not always increased in HCC[15]. Generally speaking, the higher the tumor differentiation, the lower the AFP level. However, elevated AFP levels predict HCC

Table 2 Overview of prioritization systems in different transplant regions worldwide

Region	Country	Basic listing	Standard exception	Patient benefit
Eurotransplant	Germany		1 tumor > 2 and < 5 cm up to 3 tumors > 1 and < 3 cm	Initial listing with MELD 22; upgrading every 3 mo by 10% mortality risk
	The Netherlands		1 tumor > 2 and < 5 cm up to 3 tumors > 1 and < 3 cm	Initial listing with MELD 20; upgrading every 3 mo by 10% mortality risk However, "test of time": patient must have been on the waiting list for 6 mo prior
	Austria	Possible (if Milan criteria are met); however, irrelevant with center-based allocation	No	
Europe	United Kingdom	Single lesion < 5 cm Up to 5 lesions < 3 cm Single lesion between 5 and 7 cm without progression over 6 mo No extrahepatic tumor No macrovascular invasion AFP < 1000 U/L		No prioritization on the waiting list
	France	Complex French Liver Allocation Score under consideration of Lab-MELD-Scores Tumor stage (T2 ranked higher than T1) Elapsed waiting time Distance between donor and Recipient hospital		
	Switzerland		1 tumor > 2 and < 5 cm up to 3 tumors > 1 and < 3 cm	Lab-MELD + 1.5 points per month
North America	United States		1 tumor > 2 and < 5 cm up to 3 tumors > 1 and < 3 cm	Initial listing with MELD 22; upgrading every 3 mo by 10% mortality risk
South America	Brazil		1 tumor < 5 cm up to 3 tumors of less than 3 cm each	Initial listing with 20 points, increase to 24 points after 3 mo and 29 points after 6 mo

recurrence in a multi-predictive model, together with elevated liver enzymes, lactate dehydrogenase, small resection margins, and advanced tumor stage[16]. In an analysis of approximately 1500 patients with liver cirrhosis, AFP levels showed a sensitivity of 99% and a specificity of approximately 72% to detect HCC in combination with ultrasound[17]. Recently, several studies showed that during hepatitis treatment, AFP levels were helpful to detect HCC development[18-20].

The recurrence of HCC after LT is a problem; therefore, patients with a high risk of recurrence must be detected. A retrospective cohort study showed that a combination of biomarkers, such as AFP and the MC, was more sensitive for predicting tumor recurrence than the MC alone[21]. In a multivariate analysis, AFP was the only pre-transplant predictor of HCC recurrence and mortality in a cohort of 1074 patients transplanted for HCC[22]. Grąt *et al*[23] analyzed 121 patients undergoing LT for HCC and demonstrated that the combination of up-to-7 criteria and University of California, San Francisco (UCSF) criteria with AFP levels less than 100 ng/mL was associated with a minimized risk for tumor recurrence. Previously, in a small cohort of 20 patients, a cut-off level of 100 ng/mL for AFP was shown to be a predictor for HCC recurrence after LT[24].

AFP remains the gold standard biomarker for HCC detection and a prognostic marker for post-transplant tumor recurrence, particularly in combination with other morphological tumor criteria or diagnostic tools.

Response to bridging

To avoid tumor progression and waitlist drop-off, patients waiting for LT undergo local bridging therapies[25] such as transarterial chemoembolization (TACE), which has been shown to be effective and was first proposed as a treatment option in 1977[26]. TACE produces a combination of localized chemotherapy and ischemia by occluding feeding vessels with concomitant tumor necrosis[27]. Other bridging treatments are selective internal irradiation (SIRT) or radiofrequency ablation (RFA)[28,29]. The question of who is likely to benefit from locoregional therapy remains controversial, but, currently, the proposed optimal candidates for TACE have almost normal liver function, a tumor localized to the liver, an estimated survival of 16 mo[30], tumors > 3 cm in size, and signs of hypervascularization[31]. Some authors noted that poor liver function, vascular invasion, extrahepatic tumor load, bilobar tumors, arterioportal fistula, portal vein thrombosis, or renal dysfunction are contraindications for TACE[30,32]. A systematic review analyzed the different techniques and substances used for TACE[33]. Doxorubicin, epirubicin, or cisplatin alone or in combination are used as local chemotherapy. In addition to these drugs, lipiodol, gelatin sponge particles, or beads are injected after

the chemotherapeutic substance to embolize the tumor-supplying artery. This should be performed in a selective or superselective manner to avoid damage to the nontumorous liver[31,33]. Acute liver failure, acute renal failure, gastrointestinal bleeding, and abscess formation are side effects of TACE, and a treatment related mortality rate of 2.4% was reported[33], mainly because of liver failure. The timing of TACE before LT remains unclear[34]. Decaens *et al*[35] showed that TACE only had a beneficial effect in patients with a waiting time longer than 4 mo. Others showed a positive effect of TACE on survival and tumor recurrence in patients with at least 6 mo on the waiting list[34,36]. The complete response is reported to be up to 30%[37], but rates of progressive disease following treatment have been reported to be 20%[38]. Overall, several studies have demonstrated that TACE prior to LT was associated with a good response rate for advanced tumor stages with acceptable survival rates after LT of 40% to 90% after at least 4 years, even in patients with HCC outside the MC[39-43]. A recently published study investigated 204 patients with HCC undergoing LT and showed reduced survival in patients with TACE prior to transplantation. The authors concluded that this might be because of a higher amount of pulmonary and distant metastasis[44]. Although most results appear promising, there are no controlled prospective trials to investigate the benefits of TACE prior to LT; thus, this issue remains controversial.

In contrast to TACE, SIRT is more effective in cases of large and multifocal HCC[28]. Negative side effects are rare[45-47]. Portal vein thrombosis is not an absolute contraindication for selective internal irradiation and, therefore, offers an alternative to TACE[48]. Selective internal irradiation leads to downstaging or tumor size reduction in 32% to 56% of patients with HCC[49,50]. Selective internal irradiation therapy is useful in bridging patients with HCC until LT[49,51].

During local tumor therapy with RFA, the tumor is destroyed because of local hyperthermia or chemical injury. RFA is performed percutaneously or during a surgical procedure[29]. Thus far, RFA has been shown to be a safe and effective procedure as a bridging therapy prior to transplantation[29,52-55]. A combination of different bridging therapies, such as RFA and TACE, are safe and effective regarding tumor necrosis[56]. However, a Cochrane analysis demonstrated that there is a lack of randomized clinical trials and no evidence demonstrating the superiority of RFA for bridging therapy over no intervention, chemotherapy, or transplantation[57].

Positron emission tomography

Positron emission tomography (PET) measures the metabolic activity of tumor tissue. In most oncologic cases, the tracer ^{18}F-fluorodeoxyglucose (^{18}F-FDG), an analog of glucose, is used[58].

The role of ^{18}F-FDG-PET as a diagnostic tool in HCC patients is controversial. One of the first studies dealing with PET in HCC was published by Teefey *et al*[59]. In this work, ultrasound, computed tomography, magnet resonance imaging, and PET were compared. PET showed the worst sensitivity but displayed good specificity. A Chinese group noted the value of PET scans for the diagnosis of HCC lymph node metastases in a pre-transplant setting and for the detection of tumor recurrence after LT[60]. A study by Lee *et al*[61] showed an excellent predictive value for the ratio of the maximum standard uptake volume of the tumor to the maximum standard uptake volume of non-tumoral liver tissue, which predicted tumor recurrence after LT. Similar results were confirmed by several workgroups[62-66].

In non-transplant settings, ^{18}F-FDG-PET is useful for estimating tumor differentiation[67], the probability of microvascular invasion[68], the diagnosis of bone metastases[69,70], and the estimation of tumor-free or overall survival[67,71-77].

One major issue is the dependency of PET results on tumor grade. Well-differentiated tumors have nearly the same metabolic activity as the surrounding liver tissue and, therefore, have similar tracer uptake.

However, as shown above, ^{18}F-FDG-PET correlated well with microvascular invasion and post-transplant outcome. ^{18}F-FDG-PET is a good marker for the biological behavior of HCC[58]. Therefore, ^{18}F-FDG-PET appears to be a promising approach to evaluate the aforementioned findings using a prospective approach.

Metabolomics, proteomics, and transcriptomics

AFP is the standard serum biomarker for the detection of HCC. As previously mentioned, AFP is not specific for HCC, and, therefore, diagnosis on the basis of this biomarker is limited. However, until now, no other marker has replaced AFP as the new standard in routine clinical use. To overcome this dilemma, new and more sensitive markers have been investigated using metabolomic, transcriptomic, and proteomic techniques.

Metabolomics is a global unbiased analysis of biomarkers that identifies small molecular metabolites reflecting normal biological or pathological processes in biological fluids, tissues, organs, and organisms[78-81]. Metabolomics can measure the metabolite complements in living and diseased systems[81]. In contrast, proteomics is able to analyze all proteins within a biological system[82].

Thus far, several metabolites have been investigated and proposed as potential biomarkers for HCC, such as the aspartate metabolism pathway[83], 1-methyladenosine[81], and aberrant lipid metabolism[84]. Urinary liquid chromatography-hybrid triple quadrupole linear ion trap mass spectrometry (LC-QTRAP MS) revealed that butyrylcarnitine and hydantoin-5-propionic acid were markers to distinguish patients with HCC from patients with liver cirrhosis without HCC[85]. Huang *et*

al[86] showed that betaine and propionylcarnitine in tissue and serum could distinguish HCC from hepatitis or cirrhosis better than AFP alone. A combination of metabolomics and transcriptomics reported reduced cellular glucose levels and reduced metabolites of cellular energy production in HCC[87]. A proteomic analysis detected 87 differently expressed proteins in patients with early HCC recurrence involved in catalytic pathways, signal transduction, and cell organization[88] and quantified novel phosphorylation sites that might be important for tumor progression in HCC[89]. The fields of metabolomics, transcriptomics, and proteomics are still emergent fields in cancer research. For most candidate metabolites or proteins, a definite role in HCC development and tumor progression is unclear, and further investigations are required. To date, there is no clinically available biomarker for HCC detection other than AFP.

MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNA molecules that are not transcribed into proteins but are important for regulating the stability and translation of protein-coding messenger RNAs[90,91]. miRNAs were first described in 1993[92,93]; since then, hundreds of miRNAs have been identified. miRNAs play an important role in tumorigenesis[94-96]. In HCC, a number of miRNAs have been identified with partially prognostic significance[90,97,98]. These miRNAs can be downregulated, *e.g.*, miR-122[99,100] and miR-199[101,102], or up-regulated, *e.g.*, miR-21[103], miR-221[104,105], and miR-222[104,106,107]. This is only a partial list of the previously reported miRNAs. Therefore, miRNAs could be important diagnostic and therapeutic targets in the future of cancer and HCC therapy.

Circulating tumor cells

Despite the radical resection of localized HCC with hepatectomy or LT, postoperative tumor recurrence and metastasis are frequently observed[108,109], with the transplanted liver the most frequent site of early recurrence[110]. After access of the primary tumor cells to the blood stream, circulating tumor cells (CTCs) are postulated to be responsible for tumor recurrence and tumor metastasis after complete surgical resection[110-113]. Several methods have been investigated in the past to detect CTCs, mainly based on the identification of tumor-specific antigens or epithelial cell surface antigens that are present on the primary tumor[114,115]. One of the most frequently used markers for the detection of circulating tumor cells is the epithelial cell adhesion molecule, which is only expressed on a small proportion of HCC tumors[116]. In addition, in one-third of patients, only low numbers of CTCs are detectable[117]. Therefore, this technique is not suitable for the routine detection of HCC CTCs[118]. Novel approaches to detect CTCs showed promising results[119,120], but until CTC detection is able to guide the therapy of HCC patients, further basic and clinical research is required.

INTRAOPERATIVE FACTORS

Ischemia time

Intraoperative factors are less likely to be associated with tumor recurrence in the long-term. However, there is some evidence that ischemia times play a significant role in the recurrence of HCC.

A recent published study by Nagai *et al*[121] showed a significant effect of both cold (CIT) and warm ischemia time (WIT) on post-transplant HCC recurrence. In the multivariate analysis, a CIT of more than 10 h and a WIT of more than 50 min were risk factors for the development of a recurrent HCC[121]. These results were confirmed by a Munich workgroup[122].

This observation is explained by ischemia-reperfusion injury, which leads to hepatic microcirculatory barrier dysfunction and activates cell signals related to invasion and migration[121]. Furthermore, a cellular cascade leading to angiogenesis, cellular proliferation, and growth is activated by ischemia.

This theory was confirmed by an analysis by Croome *et al*[123], who showed an inferior outcome in HCC patients who received an organ from a donor who underwent cardiac death. These grafts are exposed to additional WIT.

Transfusion

Bleeding during LT remains a major problem, and sometimes large amounts of blood products are required[124-127]. Over the last decades, there has been a significant reduction in the need for transfusions[128]. Several studies have shown that blood loss and transfusion during LT were associated with decreased overall survival and increased complications[129,130]. In addition, perioperative transfusion is associated with earlier tumor recurrence and cancer-related mortality in colorectal cancer resection[131-135] and liver resection for colorectal liver metastases[136,137]. Shiba *et al*[138] showed that a reduction of blood supply during liver resection for HCC was associated with increased survival. In a meta-analysis of 5635 patients undergoing surgery for HCC, survival, tumor recurrence, and complications were negatively correlated with blood transfusion[139]. However, the use of intraoperative autotransfusion during liver surgery because of malignancy showed no negative effects in terms of survival or tumor dissemination[140,141]. Several studies have investigated the safety of blood salvage autotransfusion regarding tumor recurrence during LT in HCC patients. The authors concluded that in cases where nonruptured HCC tumor cells were filtered, or particularly when a leukocyte depletion filter was used, there was no increase in risk of tumor

recurrence[142-144].

POSTOPERATIVE FACTORS

Immunosuppression

There is a relationship between the inflammatory state and carcinogenesis[7,145]. Pro-inflammatory cells and cytokines play a pivotal role in tumor growth, tumor invasion, and tumor spread[146,147]. Therefore, immunosuppression after transplantation can modify the inflammatory state and influence tumor recurrence. Most immunosuppression has a negative effect on the outcome of patients with HCC undergoing LT. Steroids[148], basiliximab[149], and calcineurin inhibitors (CNIs)[150] are postulated to be associated with an increased risk of HCC recurrence. In contrast, several studies reported that mammalian target of rapamycin inhibitors (mTORi) have positive effects on tumor recurrence and are favored drugs in HCC patients after LT[151-153]. A meta-analysis of five studies demonstrated a decreased recurrence rate and increased recurrence-free and overall survival in patients with sirolimus-based immunosuppression compared to patients with CNIs[154]. One reason for the positive effect of mTOR could be the inhibition of the phosphatidylinositol 3 phosphate kinase (PI3K)/Akt/mTOR pathway, which is a key regulator of the cell cycle and is responsible for cell proliferation and cancer[155]. However, thus far, randomized controlled prospective studies with long-term follow-up investigating the influence of this immunosuppression treatment are lacking[156].

Adjuvant treatment

Even after the use of a potentially curative treatment for gastrointestinal tumors, adjuvant therapy is used in most cases depending on the tumor stage. For HCC, this concept was not accepted for a long time[157]. A review by Duvoux *et al*[158] identified the problem with adjuvant therapy protocols after LT: most studies were small and retrospective with a low level of evidence. The authors concluded that the homogeneous and ethical selection of patients with a high risk of recurrence, stratification by confounding factors such as pre-transplant therapies and post-transplant immunosuppression, relevant endpoints focusing on recurrence, and appropriate follow-up are key points for appropriate studies on this issue. Similar conclusions were drawn 2 years later by Fujiki *et al*[159].

Even recently published trials lack the inclusion of a sufficient number of patients. A study by Teng *et al*[160] showed a beneficial effect of sorafenib in a case-control study. However, sorafenib was used in only 11 patients with HCC beyond the MC in either a curative intervention (n = 5) or a palliative regime after LT. Another prospective trial showed a benefit of sorafenib application after LT in seven patients with HCC beyond the MC compared to a historic control group[161].

The increased toxicity of sorafenib in patients after LT should be taken into account. This increased toxicity, which is not mechanistically understood, should lead to a dose reduction in affected patients[162].

In addition to sorafenib, Licartin has been proposed as a potential adjuvant treatment for HCC[158,159]. One Chinese study evaluated Licartin, an ^{131}I-radiolabeled murine monoclonal antibody, in a transplantation setting with excellent results regarding the tumor recurrence rate and the overall survival in the treatment group[163]. Additional data or even multicenter data for Licartin after LT are unfortunately still lacking.

Some authors have used conventional chemotherapy protocols for the treatment of HCC after LT. Zhang *et al*[164] showed good short-term (1 year) results for a treatment protocol using the FOLFOX regime. The overall survival was even better in the midterm (3 years), but the recurrence rate did not differ significantly from the control after this time period. Wu *et al*[165] performed a randomized three-arm study and showed that the gemcitabine regimen and conventional chemotherapy significantly improved the survival rate and disease free survival rate of HCC patients who had major vascular invasion and/or microvascular invasion after LT compared to a best supportive care group.

In summary, the best approach for adjuvant treatment protocols has not been identified. Large, prospective, randomized studies should be performed in the future.

CONCLUSION

LT is the treatment of choice for selected patients with HCC. There are several factors that should be taken into account, particularly in preoperative settings. The previously used selection criterion of pure morphometric variables should be modified to include biological parameters, which offer a better risk stratification for tumor recurrence.

In addition to intraoperative parameters that influence the post-transplant prognosis (ischemia times, transfusion), immunosuppression is an important tool to prevent or at least reduce the recurrence of HCC. Adjuvant protocols have not yet been established for HCC.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Management of hepatitis B virus infection after liver transplantation

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Abstract

Chronic hepatitis B virus (HBV) infection is responsible for up to 30% of cases of liver cirrhosis and up to 53% of cases of hepatocellular carcinoma. Liver transplantation (LT) is the best therapeutic option for patients with end-stage liver failure caused by HBV. The success of transplantation, though, depends on receiving prophylactic treatment against post-transplant viral reactivation. In the absence of prophylaxis, liver transplantation due to chronic hepatitis B (CHB) is associated with high rates of viral recurrence and poor survival. The introduction of treatment with hepatitis B immunoglobulins (HBIG) during the 1990s and later the incorporation of oral antiviral drugs have improved the prognosis of these patients. Thus, LT for CHB is now a universally accepted option, with an estimated 5 years survival of around 85% *vs* the 45% survival seen prior to the introduction of HBIG. The combination of lamivudine plus HBIG has for many years been the most widely used prophylactic regimen. However, with the appearance of new more potent oral antiviral agents associated with less resistance (*e.g.*, entecavir and tenofovir) for the treatment of CHB, new prophylactic strategies are being designed, either in combination with HBIG or alone as a monotherapy. These advances have allowed for more personalized prophylaxis based on the individual risk profile of a given patient. In addition, the small pool of donors has required the use of anti-HBc-positive donors (with the resulting possibility of transmitting HBV from these organs), which has been made possible by suitable prophylactic regimens.

Key words: Hepatitis B virus; Liver transplantation; Recurrence; Prophylaxis; Hepatitis B immunoglobulin

Core tip: The current success of liver transplantation in patients with chronic hepatitis B (CHB)-related cirrhosis is mainly due to the use of prophylaxis with hepatitis B immunoglobulins (HBIG) and oral antivirals against post-liver transplant recurrence of CHB. The combination of low-dose HBIG plus antivirals forms the current standard prophylaxis. The use of newer antivirals (entecavir and tenofovir), coupled with better understanding of the predisposing factors for recurrence of CHB, has led to new perspectives for prophylaxis regimens, aimed at withdrawal of HBIG or the use of HBIG-free regimens, oriented toward a strategy of individualized prophylaxis.

Jiménez-Pérez M, González-Grande R, Mostazo Torres J, González Arjona C, Rando-Muñoz FJ. Management of hepatitis B virus infection after liver transplantation. *World J Gastroenterol* 2015; 21(42): 12083-12090 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i42/12083.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i42.12083

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is responsible for up to 30% of cases of liver cirrhosis and up to 53% of cases of hepatocellular carcinoma[1]. Liver transplantation (LT) is the best therapeutic option for patients with end-stage liver failure caused by HBV. The success of transplantation, though, depends on the need to receive prophylactic treatment against post-transplant viral reactivation. In the absence of prophylaxis, LT due to chronic hepatitis B (CHB) is associated with high rates of viral recurrence that negatively influence survival[2,3]. The introduction of treatment with hepatitis B immunoglobulins (HBIG) during the 1990s[4] and the later incorporation of oral antiviral drugs have led to improvements in the prognosis for these patients, resulting in LT for CHB now being a universally accepted option, with an estimated 5 years survival of around 85% *vs* the 45% survival seen prior to the introduction of HBIG[2,3,5]. The combination of lamivudine (LAM) plus HBIG has for many years been the most widely used prophylactic regimen. However, with the appearance of new more potent oral antiviral agents associated with less resistance [entecavir (ETV) and tenofovir (TDF)] for the treatment of CHB, new prophylactic strategies are being designed, either in combination with HBIG or even alone in monotherapy. This has resulted in the development of more personalized prophylaxis based on the individual risk profile of a given patient[6]. In addition, the small pool of donors has required the use of anti-HBc-positive donors (with the resulting possibility of transmitting HBV from these organs), which has been made possible by suitable prophylactic regimens[7]. Table 1 reflects the risk of recurrence of hepatitis B in recipients of anti-HBc-positive organs according to the serological status of the recipient.

Table 1 Risk of de novo hepatitis B in recipients of anti-HBc-positive organs

Recipient status	Naive	AntiHBc$^+$ AntiHBs$^-$	AntiHBc$^+$ AntiHBs$^+$	AntiHBc$^-$ AntiHBs$^+$
No prophylaxis	> 40%	13%	< 2%	10%
With prophylaxis	12%	< 4%	< 2%	< 2%
	High risk	Intermediate risk	Low risk	Intermediate risk

PROPHYLAXIS FOR HBV RECURRENCE AFTER LT

Various strategies have been suggested to aid in the prevention of HBV recurrence after LT.

HBIG monotherapy

HBIG was the first effective drug used as prophylaxis for recurrence of CHB in transplant patients. It led to great advances, as it reduced the rates of recurrence to around 20%-30% and significantly improved survival rates[4]. However, the use of HBIG as prophylaxis in monotherapy has certain complications, such as the potential for mutations in the surface gene that determines resistance and loss of efficacy, the inability to reach sufficiently protective anti-HBs titers in all patients[8,9], and the high economic cost and difficulties associated with their parenteral administration[10]. These inconveniences, together with the appearance of new oral antiviral nucleos/tide analogues (NAs) and the confirmation of their synergistic effect, mean that HBIG is no longer used in monotherapy, and the standard treatment for prophylaxis against CHB recurrence is now combined therapy with HBIG plus NAs[10].

NAs in monotherapy

LAM: LAM was the first effective oral antiviral used against CHB. Its safety and efficacy, even in patients with decompensated hepatic cirrhosis, enabled patients to have a negative viremia at LT, thus reducing the probability of post-LT viral recurrence[11].

Perrillo *et al*[12], using LAM monotherapy both before and after LT, reported a recurrence rate of around 30%, very similar to that seen with HBIG monotherapy[4]. Furthermore, it was also noted that most recurrences were due to the development of HBV DNA polymerase mutations that led to drug resistance. These patients who experienced recurrence had higher viral loads at the time of LT than those who did not have recurrence, similar to patients treated with HBIG monotherapy.

Because of the high rate of resistance with LAM after prolonged use the resulting risk of recurrence, and the introduction of ETV and TDF with their high genetic barrier to resistance, LAM monotherapy has fallen into disuse.

Adefovir: The commercialization of adefovir (ADV) in 2003 represented an alternative for patients with resistance to LAM. Schiff *et al*[13] studied a group of 60 patients, of whom 24 received ADV with or without LAM with no HBIG as prophylaxis against post-LT hepatitis B, and found that none developed recurrent hepatitis B after a follow-up of 36 mo. However, because of the potential nephrotoxic effect associated with ADV and the risk of developing resistance ADV is not the first choice for prophylaxis against post-LT CHB recurrence.

ETV and TDF: The recent availability of these highly effective, well-tolerated antivirals with their high genetic barrier to resistance has resulted in changes in the approach to CHB in relation to LT. Accordingly, prophylactic strategies are now being reconsidered (see below).

Combined prophylaxis with HBIG plus oral antivirals

Various studies and meta-analyses[14-18] on the synergic effect of combination HBIG and NAs in prophylaxis for CHB recurrence have found general recurrence rates < 10%, which is noticeably lower than those seen with HBIG or NAs in monotherapy. This reduction in recurrence has led to combined prophylaxis (mainly HBIG plus LAM) becoming the standard of care in LT due to CHB. The possibility of resistance with long-term use of LAM encouraged a trial on the use of combination HBIG and ADV. A systematic review by Cholongitas *et al*[19] found that the combination of HBIG plus ADV was more effective than prophylaxis with HBIG plus LAM (2% *vs* 6.1%, $P = 0.024$).

However, relatively few studies have examined combined prophylaxis with HBIG plus ETV or TDF. Overall, these studies have found recurrence rates ranging from 0% to 4%[20-26]. Nevertheless, no randomized studies have yet compared the efficacy of combined prophylaxis with HBIG + LAM *vs* HBIG + ETV or TDF, although a recent systematic review noted a higher recurrence rate with the combination HBIG + LAM than with HBIG + ETV/TDF (6.1% *vs* 1%, $P = 0.0004$)[27].

Prophylaxis with alternative dosing schedules of HBIG

The high economic cost of prophylaxis schedules combining HBIG plus oral antivirals, together with the high efficacy and safety of the more recent oral antivirals (ETV/TDF), have led to the study of different prophylactic strategies aimed at lowering or eliminating HBIG in order to reduce costs and the inconveniences associated with its administration.

Additionally, other possible routes of administration of HBIG have been assessed. Several studies[28,29] have shown that low-dose intra-muscular (IM) administration of HBIG when combined with NAs is a cost-effective alternative to its intravenous (IV) administration. Recently the subcutaneous administration of HBIG has been found equally effective, well-tolerated, and accepted by patients[30,31].

HBIG dose reduction: Two differing strategies have been tried: (1) Low-dose IM administration of HBIG (400-500 IU) at fixed intervals with oral antivirals. Two studies endorse these results; Gane *et al*[29] found recurrence rates of 1% during the first year and 4% in the fifth year, using doses of 400-800 IU per day for the first week and then monthly in combination with LAM. Zheng *et al*[32] used doses of 800 IU at first weekly and then monthly, recording recurrence rates of 15% at 2 years. In both studies, the rates of recurrence were significantly higher in those patients with HBV-DNA values > 10^5 copies/mL at the time of transplantation; and (2) "On demand" use of HBIG doses to maintain anti-HBs titers between 50-100 IU/L, considered protective when administered together with oral antivirals. Although this strategy can be more cost effective, it requires repeated monitoring of anti-HBs titers, as the amount of HBIG needed to reach a certain level of anti-HBs varies greatly from patient to patient. Using this strategy plus LAM, Jiang *et al*[33] found recurrence rates of 2.3% the first year, 6.2% the third year, and 8.2% the fifth year. As before, pretransplant HBV-DNA levels > 10^5 copies/mL were associated with greater recurrence.

These studies[29,32,33], therefore, show that IV administration of high doses of HBIG is neither necessary nor cost-effective when given together with oral antivirals and also highlight the importance of pretransplant levels of HBV-DNA as a predictive factor for recurrence.

Withdrawal of HBIG after combined prophylaxis: Studies of this strategy vary greatly in design, type of antiviral agent used, and time from LT to HBIG withdrawal. In addition, most of the studies are observational and from a single center[34-44], with just three randomized studies[45-47]. The overall rates of recurrence in these studies ranged from 0% to 17%[34-47].

Using this strategy, it is necessary to note that although the results during the initial years after withdrawing HBIG are good, the risk of recurrence can increase over time due to the appearance of resistance and, in particular, to lack of adherence[48]. The problem of the appearance of resistance may be of little importance if high genetic barrier oral antivirals are used, such as ETV or TDF. To date, only four studies have been published[34,37,42,43], none randomized, analyzing recurrence after withdrawal of HBIG using

TDF or ETV. A systematic review by Cholongitas *et al*[27] that included the patients in these four studies found a recurrence rate of 3.9% *vs* 1% in the case of combined prophylaxis with HBIG and ETV/TDF, although the difference was not significant (P = 0.17).

This therapeutic strategy seems to be associated with a greater risk of recurrence in those patients with high HBV-DNA levels at the time of transplantation. One study found that detection of low and transitory HBV-DNA levels was not necessarily associated with recurrence, and only those patients who had persistently high hepatitis B surface antigen (HBsAg) and/or HBV-DNA levels had a high risk of experiencing recurrence[44].

Prophylaxis without HBIG: Experience using regimens of prophylaxis without HBIG and just oral antivirals is very limited[25,49-54]. Fung *et al*[52] studied 80 patients who received ETV monotherapy as prophylaxis, and the rate of HBsAg positivization was 22.5%, where only one patient (1.2%) was positive for HBV-DNA after the 26 mo follow-up period. Likewise, they found that the patients with HBV-DNA < 5 log copies/mL and HBsAg values < 3 log IU/mL pretransplant had an accumulated rate of HBsAg negativization at 18 mo of 100% *vs* 78% in the patients who did not fulfill these criteria. A more recent study by these same authors[53] showed that recurrence in patients treated with ETV (n = 142) was 0% at 3 years, whereas recurrence in those patients treated with LAM (n = 176) was 17% (P < 0.001). A study by Wadhawan *et al*[49] using different antivirals in regimens without HBIG (ETV, n = 42; LAM + ADV, n = 19; TDF, n = 12; and ETV + TDF, n = 2) noted recurrence (defined as HBV-DNA positivity) in 6/75 (8%) patients, five of these related to lack of treatment adherence. Cholongitas *et al*[34], in a systematic review, noted a significantly higher recurrence rate among patients who received prophylaxis completely free of HBIG, using ETV or TDF, compared with those who received combined therapy with HBIG plus LAM, with recurrence defined as HBsAg positivity [26% (29/112) *vs* 5.9% (109/1834), P < 0.0001]. Considering recurrence as HBsAg positivity and detectable DNA, the recurrence rates were 0.9% with HBIG-free therapy *vs* 3.8% with combined therapy, although the difference was not statistically significant (P = 0.11). No differences were found in relation to the antiviral used or the use of double-antiviral prophylaxis.

No studies are yet available concerning the combined use of two ANs (*e.g*., ETV + TDF or TDF plus emtricitabine) as prophylaxis without HBIG.

Vaccination against HBV

Active immunization with recombinant anti-HBV vaccines could be an attractive alternative to the indefinite administration of HBIG, particularly in patients with a low risk of recurrence. However, the few studies available provide contradictory results; and, at the present time, their generalized use cannot be recommended, at least as an isolated prophylactic strategy for post-LT CHB[55,56].

Individualized prophylaxis against HBV

In recent years, growing scientific evidence supports the possibility of reducing or even completely withdrawing HBIG from prophylaxis regimens against post-LT HBV, especially in patients with a low risk of recurrence. When considering the prophylactic regimen, it is necessary to consider all those factors that may affect viral recurrence, including virus-dependent factors [DNA-HBV and HBsAg levels at the time of transplantation, antiviral resistance, coinfection with hepatitis delta virus (HDV), human immunodeficiency virus], patient-related factors (treatment adherence, coexistence of hepatocarcinoma), or those related with the particular antiviral used (antiviral potency, genetic barrier). The pretransplant DNA-HBV levels and the existence of antiviral resistance are considered the most important predictive factors for post-transplant recurrence[29,53,57]. The presence of pretransplant hepatocarcinoma, especially if there is post-transplant recurrence of CHC, is associated with a greater risk of HBV recurrence[58,59]. On the other hand, fulminant hepatitis B is associated with a low risk of HBV recurrence[60], as is coinfection with HDV[10].

Thus, those patients considered to be at low risk for recurrence (negative pretransplant viremia, prior absence of antiviral resistance) could be considered for HBIG-free prophylaxis or with HBIG just given for a limited time (1-6 mo), using antivirals with a high genetic barrier (ETV/TDF) and provided there are no treatment adherence problems. On the other hand, patients at high risk for recurrence, as well as those with limited options for treatment if prophylaxis fails (*e.g*., patients with delta coinfection) would benefit more from a long-term regimen based on the combination of HBIG plus antivirals.

ANTI-CORE-POSITIVE DONORS

The imbalance between the high demand for transplant organs and the paucity of donors has necessitated the use of serum HBV-positive organs (anti-HBc positive, HBsAg negative). These represent the main risk factor for the *de novo* development of hepatitis B in transplant patients[7]. Several studies have shown equal survival for anti-HBc positive and anti-HBc negative organ recipients[61-63]. The risk of developing *de novo* hepatitis B in anti-HBc-positive organ recipients depends mainly on the serological status of the recipient at the time of transplantation and the adoption of effective prophylactic measures. A systematic review by Cholangitas *et al*[7] found that if recipients were negative for both anti-HBc and anti-

HBs the risk of recurrence was 48%, if the recipient was anti-HBc positive the risk was 13%, and if the recipient was anti-HBc positive and anti-HBs positive, the risk was reduced to < 2%. With prophylaxis, the risks were 12%, < 4%, and < 2%, respectively (Table 1). This prophylaxis is specifically for: (1) patients with no immunity against the virus (HBsAg negative, anti-HBc negative, anti-HBs negative); (2) patients with acquired immunity (vaccinated: anti-HBs positive, anti-HBc negative and HBsAg negative); and (3) patients who are anti-HBc positive and anti-HBs negative. Prophylaxis is not generally advisable in patients with natural acquired immunity (HBsAg negative, anti-HBc positive with anti-HBs positive) given the minimum or null risk of reinfection[7,64].

Prophylactic strategies for *de novo* hepatitis B in recipients of anti-HBc organs have traditionally consisted of HBIG with or without LAM. Diverse studies have shown that HBIG monotherapy is inferior to that of HBIG combined with LAM, and that combination therapy with HBIG and LAM is no more efficient than monotherapy with LAM to prevent resistance[7]. The systematic review by Cholongitas *et al*[7] found that the rate of *de novo* hepatitis B with monoprophylaxis with LAM was < 3%. The high cost of prophylaxis with HBIG and the introduction over recent years of new antivirals with a high genetic barrier (ETV/TDF) has resulted in many transplant centers ceasing to use HBIG as passive prophylaxis[7,64,65]. Nevertheless, the heterogeneity of studies concerning prophylactic strategies with the use of anti-HBC positive organs necessitates large, well-designed multicenter studies to provide a high level of scientific evidence.

TREATMENT OF POST-LIVER TRANSPLANT HEPATITIS B

Hepatitis B in the LT patient can appear through transmission from a risk contact, transmission from an anti-HBc positive organ with no adequate prophylaxis (*de novo* hepatitis B), or by reactivation of a prior hepatitis B when prophylaxis fails (recurrent hepatitis B). Whatever the reason, treatment of established hepatitis B in the LT patient is based on the same recommendations as for immunocompetent patients.

The most commonly accepted definition of recurrent hepatitis B is the reappearance of circulating levels of HBsAg after transplantation, with or without HBV-DNA positivization or histological evidence of disease. Nonetheless, a few authors, such as Lenci *et al*[66], suggested that the definition of recurrence should include one or more of the following at some time after transplantation: (1) HBsAg positivity; (2) detectable serum levels of HBV-DNA; (3) detectable levels of covalently closed circular DNA in liver tissue; (4) increase in alanine aminotransferase; or (5) liver damage seen in a liver biopsy. Whilst this definition would seem more useful from the practical point of view, it is not yet universally accepted. Those patients who, years after transplantation, are still HBsAg-positive with negative HBV-DNA may have a high risk for a clinical, histological, and biochemical recurrence of hepatitis B. Accordingly, it is not advisable to stop HBIG in these patients.

CONCLUSION

LT for patients with hepatic cirrhosis due to CHB is now a universally accepted treatment. The magnificent results have been made possible in great measure by the use of HBIG and oral antivirals for prophylaxis against post-LT recurrence of CHB. The combination of low-dose HBIG plus antivirals is now considered the standard prophylaxis for post-LT recurrence of hepatitis B. The use of the newer antivirals (ETV and TDF), together with our better understanding of the factors that predispose patients to recurrence of CHB, has allowed for new focus to be placed on prophylaxis regimens. These efforts are aimed at withdrawing HBIG or the use of HBIG-free regimens from the outset, using only oral antivirals, especially in patients at low risk of recurrence, thus applying a strategy of individualized prophylaxis. In addition, the efficacy of these prophylactic regimens has enabled the use of grafts from anti-HBc positive donors without these being considered a risk, thereby increasing the donor pool.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Application of nucleoside analogues to liver transplant recipients with hepatitis B

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Abstract

Hepatitis B is a common yet serious infectious disease of the liver, affecting millions of people worldwide. Liver transplantation is the only possible treatment for those who advance to end-stage liver disease. Donors positive for hepatitis B virus (HBV) core antibody (HBcAb) have previously been considered unsuitable for transplants. However, those who test negative for the more serious hepatitis B surface antigen can now be used as liver donors, thereby reducing organ shortages. Remarkable improvements have been made in the treatment against HBV, most notably with the development of nucleoside analogues (NAs), which markedly lessen cirrhosis and reduce post-transplantation HBV recurrence. However, HBV recurrence still occurs in many patients following liver transplantation due to the development of drug resistance and poor compliance with therapy. Optimized prophylactic treatment with appropriate NA usage is crucial prior to liver transplantation, and undetectable HBV DNA at the time of transplantation should be achieved. NA-based and hepatitis B immune globulin-based treatment regimens can differ between patients depending on the patients' condition, virus status, and presence of drug resistance. This review focuses on the current progress in applying NAs during the perioperative period of liver transplantation and the prophylactic strategies using NAs to prevent *de novo* HBV infection in recipients of HBcAb-positive liver grafts.

Key words: Nucleoside analogues; Liver transplantation; Hepatitis B virus; Hepatitis B immunoglobulin; Hepatitis B core antibody positive donors; *de novo* hepatitis B; Prophylactic regimen

Core tip: Hepatitis B virus (HBV)-related end-stage liver disease is a common indication for liver transplant. This

review discusses application of nucleoside analogues (NAs) for patients on liver transplant waiting lists, as well as the preventive and therapeutic strategies of NAs for HBV recurrence post-transplantation. The prophylactic role of NAs for recipients of livers from HBV core antibody-positive donors is also discussed. This review will help physicians and surgeons improve management of HBV-related liver transplant patients.

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INTRODUCTION

Hepatitis B virus (HBV) infection is the most common chronic viral infection worldwide and ranks as one of the top health burdens[1]. Serological evidence of current or past HBV infections is found in about 30% of the world's population[2,3]. Despite the development of management and treatment for chronic HBV infection, some patients still advance to end-stage liver disease, where liver transplantation is the only treatment. In the early 1980s, chronic HBV was regarded as a contraindication for liver transplantation since the results were disappointing, with graft reinfection rates at 80%-100% and two-year graft and patient survival at approximately 50%[4-6].

In the late 1980s, nucleoside analogues (NAs) and hepatitis B immune globulins (HBIG) were introduced as new prophylaxis strategies against the recurrence of HBV following liver transplantation. As a result, survival rates increased to over 75% and reinfection rates fell to less than 10% after 5 years[7-9]. In this combination therapy, NAs suppress the replication of HBV and reduce the damage to hepatocytes by hindering the synthesis of reverse transcriptase (RT), which is essential for viral replication[10]. HBIG is a polyclonal antibody to HBV surface antigen (HBsAg), which performs a central role in prophylaxis against recurrent hepatitis B in liver transplant recipients[11]. However, the use of HBIG is undermined by its high cost.

Several studies have aimed to minimize or eliminate the use of HBIG without sacrificing low HBV recurrence rates. Today, NAs remain the key therapy for HBV infection in liver transplant recipients.

CLASSIFICATION AND CHARACTERISTICS OF NAs

Classification of NAs

Three main groups of NAs are used, which are based on their structural classification. The approved NAs include lamivudine (LAM), a deoxycytidine analog with an unnatural L conformation, and the related L-nucleoside, telbivudine (LDT). The second group, the acyclic phosphonates, includes adefovir dipivoxil (ADV), a prodrug for the acyclic 20-deoxy adenosine monophosphate analog adefovir, and the structurally similar tenofovir (TDF). The third group contains a D-cyclopentane sugar moiety and includes the most potent anti-HBV drug discovered to date, the deoxyguanosine analog entecavir (ETV)[12]. LAM was the first oral antiviral agent against HBV, and initially showed many positive efficacy and tolerability effects. However, it is no longer the treatment of choice due to its high risk of resistance[13,14]. ADV used to be prescribed for patients with LAM resistance[15], but they too faced development of resistance against ADV, and better NA alternatives were developed. ETV and TDF are newer antiviral drugs and have superseded other NAs since they have superior antiviral efficacy and are resilient to resistance. For these reasons, ETV and TDF are currently recommended as the first-line therapy for HBV-infected patients[16].

Drug resistance of NAs

Although NAs can suppress the replication of HBV and control the progression of the disease, drug resistance has limited their long-term effectiveness. Although HBV has a DNA genome, it replicates through reverse transcription of an RNA intermediate. The lack of a proofreading ability of the virally-encoded RNA-dependent DNA polymerase can result in mutations at multiple nucleotide positions in the genome[17,18]. Viral fitness, potency, and genetic barrier to resistance of the antiviral agents are the major factors associated with the development of antiviral resistance[19]. For liver transplant recipients, the goal of antiviral therapy is to prevent the reinfection of HBV following transplantation. However, the development of drug resistance is a common problem for such patients because of the need for long-term NA use. It is therefore important to optimize the duration of use, type, and dose of NAs before and after liver transplantation. A summary of the structure, mechanism of action, and incidence of resistance of currently available NAs is listed in Table 1[12,20,21].

USAGE OF NAs FOR PATIENTS ON LIVER TRANSPLANT WAITING LISTS

Purpose of antiviral therapy in pre-transplantation patients

Every patient with chronic HBV infection is potentially infectious and at risk of developing liver complications. Those who develop complications usually undergo antiviral therapy. However, current medications rarely achieve viral eradication in patients with chronic HBV infection[22]. Due to the development of antiviral

Table 1 Structure, mechanism of action, and incidence of resistance of currently available nucleoside analogues[12,20,21]

Drug name	Structure	Mechanism of action	Incidence of drug resistance
LAM	Cytidine analogue	Premature chain termination	High
LDT	Thymidine analogue		High
ETV	Guanosine analogue	Inhibits priming RT Inhibit negative and positive strand synthesis	Low
ADV	Adenosine analogue	Premature chain termination	High
TDF		Inhibit negative strand synthesis	Unknown

ADV: Adefovir dipivoxil; ETV: Entecavir; HBV: Hepatitis B virus; LAM: Lamivudine; LDT: Telbivudine; NA: Nucleoside analogue; RT: Reverse transcriptase; TDF: Tenofovir.

resistance, many patients treated with antiviral drugs nevertheless progress to liver cirrhosis. Once patients develop decompensated cirrhosis, liver transplantation is the only method of treatment available. The goal of antiviral therapy for those who eventually undergo liver transplantation is to decrease the risk of HBV re-infection. Virus levels should be tested every 3 mo[23]. Antiviral therapy in pre-transplantation patients is important to reduce viral load to low or non-detectable HBV DNA serum levels[24,25]. All current data suggest that an effective pre-transplantation anti-HBV therapy prevents post-transplantation HBV recurrence[26]. The appropriate treatment with NAs to HBsAg-positive patients can maintain undetectable HBV DNA, ameliorate liver injury, and improve long-term survival following liver transplantation[11,27,28]. Physicians should also consider how antivirals are used at other phases of the transplantation process, including (1) those on the waiting list; (2) prophylaxis therapy for transplant recipients; and (3) treatment of recurrent HBV when prophylaxis therapy fails[23].

LAM and ADV

LAM is the most widely used NA worldwide, due to its low cost. Its efficacy has been confirmed to improve liver function, diminish the incidence of hepatocellular carcinoma[29], and reduce the need for a liver transplant[30,31]. However, long-term LAM monotherapy is associated with an increased rate of viral resistance due to YMDD mutations (tyrosine-methionine-aspartate-aspartate mutations), which lead to treatment failure and clinical deterioration. Mutation rates are as high as 70% after 5 years of treatment with LAM[32,33]. ADV is effective against both wild type and LAM-resistant HBV strains[34]. It also improves liver function in patients with HBV-related decompensated cirrhosis[35]. However, its low potency, moderate risk of resistance during long-term therapy, and higher cost tend to impede its widespread use[29]. Furthermore, ADV has been found to be associated with adverse renal effects[36,37]. These drawbacks have fueled research efforts to develop replacements for ADV and LAM. The ideal pre-transplantation therapy will be potent, have a high genetic barrier to resistance, have good virological responses, and show long-term efficacy[38,39].

ETV and TDF

ETV and TDF are potent antiviral agents with minimal to zero risk of resistance. They are therefore currently recommended as the first line of NAs for pre-transplant therapy for patients with HBV-related decompensated cirrhosis[16]. ETV has a high genetic barrier to resistance in nucleoside-naïve patients[40]. A recent study of chronic HBV patients with hepatic decompensation showed that ETV administration significantly decreased mortality. Patients treated with ETV for 24 wk showed a greater reduction in serum alanine aminotransferase levels, which is elevated during acute exacerbation of HBV. Further, these patients had lower model for end-stage liver disease (commonly known as MELD scores) compared to those treated with LAM[41]. Zhang *et al*[42] then reaffirmed that ETV decreases MELD scores and reported that it significantly reduces HBV DNA levels and improves the long-term survival rate in HBV patients with spontaneous acute-on-chronic liver failure. In a retrospective study of patients with chronic HBV, ETV was associated with a significantly lower risk of death compared with LAM[43]. These findings support the use of ETV over other treatments for patients on the transplant waiting list. TDF is the most recent NA to be approved for chronic HBV treatment. Recent research showed that treatment with TDF monotherapy for 5 years led to prolonged virological remission in the vast majority of patients[6]. Further, TDF was found to suppress LAM- and ADV-resistant HBV, suggesting that TDF may be an effective treatment for patients who had previously experienced drug resistance[44]. Due to TDF's high potency and higher genetic barrier, it has also been used on patients with advanced liver fibrosis[45]. However, TDF has been observed to cause adverse renal effects after 1 year of treatment[46,47], and ETV has been reported to cause lactic acidosis in patients with severe liver dysfunction[48]. Studies with larger cohorts did not find any lethal complication with either ETV or TDF[32]. Despite the higher cost and potential adverse effects of ETV and TDF, they are currently recommended as the first-line therapy for chronic HBV patients and for patients with decompensated cirrhosis awaiting a new liver.

APPLICATION OF NAs IN LIVER TRANSPLANT RECIPIENTS

Prophylactic regimen with HBIG monotherapy

The use of HBIG following a liver transplantation

was the first milestone in the prevention of post-transplantation HBV recurrence. HBIG monotherapy reduced HBV recurrence by approximately 70%[49]. HBIG monotherapy with high doses (10000 IU) are typically used during the anhepatic phase, and daily doses (10000 IU) are given for the first few days following transplantation. Subsequent doses are administered according to serum HBV titers, and can be either short term (6 to 12 mo) or indefinite[50]. Long-term HBIG monotherapy is generally well tolerated. However, due to the possibility of late reinfection and mutations of cell surface genes, HBIG monoprophylaxis may not be a successful solution. For this reason, combination therapy with HBIG and NAs was introduced[51].

Prophylactic therapy with LAM plus HBIG

The second milestone for preventing HBV recurrence following liver transplantation was the approval of the use of LAM. By combining HBIG with LAM, over 95% of HBV recurrence could be prevented[52]. However, this kind of treatment also causes several problems. For example, due to the prominent risk of viral resistance, LAM is not considered as an optimal first-line choice[53]. Also, since HBIG is a type of passive immunization, meaning its effects are immediate and transient, antibody titer must be monitored to guide the therapy. Further, the high cost and inconvenient mode of HBIG administration (*i.e.*, repeated injections) represents a major burden in its use. Many use HBIG as treatment for a finite period of time in parallel with one or two NAs, while some clinicians completely eliminate HBIG in prophylactic regimens[54].

Discontinuation of HBIG

A study by Buti *et al*[55] investigated the risk of HBV recurrence following the discontinuation of HBIG therapy in patients also receiving LAM maintenance therapy. Patients were randomized into either a LAM monotherapy group or an HBIG + LAM group and treated for 1 mo. After 91 months, the two groups had similar recurrence rates of > 10%. In a retrospective study, 132 HBsAg-positive liver transplant recipients received either LAM + HBIG (n = 97) or other NA besides LAM + HBIG (n = 35). HBV recurrence was only observed in the LAM + HBIG group at 1752 d post-treatment[56]. Other studies have focused on using more effective NAs, other than LAM, to replace HBIG. Since ADV is a key therapeutic alternative for LAM-resistant patients[57], it has also been used in combination with LAM to replace HBIG. A multicenter, prospective study showed that all patients who received LAM and ADV after HBIG discontinuation had no HBV recurrence 22 mo after transplantation. Combination therapy with LAM and ADV was therefore deemed safe and effective for HBV recurrence[58]. Similarly, Nath *et al*[59] reported that no recurrence took place following 7 d of high-dose HBIG treatment (10000 IU) followed by LAM and ADV combination treatment. Further, these types of combination therapies are both more convenient and cheaper than using HBIG with LAM[60,61].

As discussed earlier, ETV and TDF are particularly suitable for patients who are resistant to LAM and ADV. One study analyzed patients with undetectable HBV DNA and HBsAg-negativity at the time of liver transplantation (n = 29). These patients were treated with HBIG and ETV for 12 mo, after which the HBIG treatment was discontinued. After 31 mo, only one patient had recurrence[62]. Teperman *et al*[63] recruited patients treated with emtricitabine (a nucleoside reverse transcriptase inhibitor)/TDF and HBIG for at least 24 wk and randomized them into two groups. One group (19 patients) received emtricitabine/TDF combined with HBIG, while the other group (18 patients) was given emtricitabine/TDF alone for an additional 72 wk. None of the study participants experienced HBV recurrence following the 72 wk in either group. After 26 mo of combined therapy with emtricitabine/TDF after HBIG discontinuation, Wesdorp *et al*[64] reported that only one out of 17 patients was HBsAg-positive (but without detectable levels of HBV DNA). Stravitz *et al*[65] reported similar results, where emtricitabine/TDF was used as a prophylaxis against HBV reinfection after HBIG discontinuation. In a recent study, liver transplant recipients who received combination therapy with HBIG and TDF or ETV were switched to NA monotherapy (ETV or TDF) for 6 mo. No recurrence of HBV was detected[66]. Cholongitas *et al*[67] evaluated the risk of HBV recurrence after withdrawal of HBIG in liver recipients who underwent HBIG/NA combination therapy for at least 12 mo. Patients without HBV recurrence were enrolled for HBIG discontinuation. Of these, 28 patients continued on LAM in combination with ADV or TDF, 10 continued on with TDF monoprophylaxis, and 9 continued on with ETV monoprophylaxis. Although three patients developed detectable HBsAg, all of them had undetectable HBV DNA and no clinical signs of HBV recurrence.

Most of the studies examining HBIG-free therapy withdrew HBIG after several months of its application. Studies focusing on a complete HBIG-free prophylactic approach immediately following liver transplantation are limited. However, one study reported on 80 liver recipients who were given ETV without HBIG as the primary prophylaxis against HBV recurrence. Ten patients tested positive for HBsAg, and eight of those remained HBsAg-positive after 26 mo. ETV-related viral resistance was not detected in these patients[54,68]. In another study, 75 patients with negativity for HBV DNA at the time of liver transplantation were enrolled. A combination of LAM and ADV was administered to 19 patients, 42 were given ETV, 12 received TDF, and 2 received a combination of ETV and TDF after transplantation. Only six patients were positive for HBV

DNA after 21 mo, five of which had stopped taking oral antiviral medication[69]. Although these studies showed that maintenance therapy with effective NAs after discontinuation of HBIG prophylaxis were effective, further studies in larger cohorts with longer follow-up periods are still needed. Studies that focus on long-term use of NAs without HBIG immediately after liver transplantation are also warranted.

Is HBIG still necessary in the prophylactic regimen with the development of NAs?

The studies described above show that in select patients, HBIG can be successfully discontinued or even excluded. However, although cost and convenience for patients have been the driving force for limiting or eliminating HBIG, it is important to realize that we should not compromise the prevention of disease recurrence. A recent systemic review showed that the application of HBIG reduced HBV recurrence and virus mutants. It also improved 1-year and 3-year survival rates. A sub-group analysis showed that patients with positive pre-transplant HBV DNA status, HBIG was necessary to reduce HBV recurrence rate[70]. Thus, only patients with an undetectable HBV DNA level prior to transplantation are suited for an HBIG-free strategy. Further, such a strategy is only possible with antiviral treatments that have high efficacy and a high genetic barrier to resistance. For those patients with high pre-transplantation HBV DNA levels or those with limited antiviral options, HBIG-free prophylaxis is not recommended[11,71]. The duration of prophylaxis should also be considered. It has been thought that adequate prophylaxis must be life-long since NAs cannot eliminate the genomic template of covalently closed circular DNA (cccDNA). As a result, NAs do not eliminate the risk of viral replication in grafts that contain cccDNA[1]. Consequently, the use of NAs does not eliminate the need for long-term prophylaxis in recipients at risk for recurrent HBV[9].

RECURRENCE OF POST-TRANSPLANTATION HBV AND ITS TREATMENT STRATEGIES

Causes of HBV recurrence after liver transplantation

With the development of new antiviral agents and appropriate prophylaxis strategies, the recurrence rate of HBV following liver transplantations has significantly decreased in the last decades. However, a 0% recurrence rate has still not been achieved. Several factors contribute to the recurrence of HBV following a liver transplantation. First, pre-transplantation patients with serum HBV DNA $\geqslant$ 105 copies/mL have a much higher rate of recurrence compared with those whose HBV DNA is undetectable. Theoretically, undetectable HBV DNA should be achieved in all patients on the waiting list before they get a new liver[72]. However, patients that are admitted with severe decompensated cirrhosis or life-threatening complications receive transplants out of urgency, regardless of HBV DNA levels. Second, liver transplant recipients receive immunosuppressant drugs to prevent transplant rejection. It is well known that any type of immunosuppressive therapy can lead to HBV reactivation[38,73]. Third, drug resistance is always a large risk in anti-HBV therapy-particularly for immunosuppressed patients. It was reported that the LAM resistance is detected in 45% of immunosuppressed patients within the first year following liver transplantation[10,74], and resistance to HBIG has also been recently reported[75,76]. Lastly, the compliance of patients also plays a role in recurrence of HBV after transplantations, where low compliance increases the risk of drug resistance[77].

Treatment strategies for HBV recurrence

Treatment approaches for recurrent HBV in liver recipients is largely based on what we learn about drug resistance in non-transplantation patients. ADV was shown to be an effective rescue therapy for patients with LAM-resistant HBV following liver transplantation[78,79]. In a retrospective study, five HBV-recurrent liver transplant recipients underwent safe and effective ADV therapy[80]. ETV and TDF are also considered rescue therapy drugs for recurrent HBV following liver transplantation. ETV has been demonstrated to be safe and effective in prophylaxis treatment, even in combination with immunosuppressive agents[81]. Fung *et al*[68] reported that although only 26% of patients achieved complete viral suppression at the time of transplantation, 98.8% of patients reached undetectable HBV DNA levels with ETV alone following liver transplantation. In a case report, the combined treatment of LAM and TDF effectively suppressed HBV DNA replication in a liver recipient who had LAM, ADV, and HBIG resistance[82]. Karlas *et al*[83] reported that combination therapy with ETV and TDF may be a promising therapy for preventing post-liver transplantation HBV recurrence. The optimal treatment method for HBV recurrence following liver transplantation is still under debate. More clinical studies are needed to thoroughly investigate ideal combinations of antiviral agents. The current treatment selections for liver transplant recipients with HBV recurrence and drug resistance are shown in Table 2[10,16,84].

USE OF HBcAb-POSITIVE LIVER DONORS AND THE PROPHYLACTIC REGIMEN FOR *DE NOVO* HBV

Use of HBcAb-positive donors

Liver transplantations have saved thousands of patients worldwide with end-stage liver diseases. However,

Table 2 Treatment selections for liver transplant recipients with HBV recurrence and drug resistance[10,16,84]

HBV recurrence after liver transplant	LAM resistance	Add ADV or switch to ETV or TDF
	ADV resistance	Switch to TDF
	ETV resistance	Switch to TDF
	HBIG resistance	Switch to NAs monotherapy or combined therapy

HBV: Hepatitis B virus; LAM: Lamivudine; ADV: Adefovir dipivoxil; ETV: Entecavir; TDF: Tenofovir; HBIG: Hepatitis B immune globulin; NA: Nucleoside analogue.

shortages of donor livers limit their use and impede transplantation research. Thus, several strategies, including the use of HBsAg-negative/HBcAb-positive donors, have been used to expand the liver donor pool. HBcAb-positive donors were previously exposed to HBV, but did not develop chronic infection[85,86]. The presence of persistent intrahepatic HBV cccDNA in HBcAb-positive donors constitutes a potential reservoir for viral reactivation[87]. In the absence of prophylaxis, the frequency of HBV transmission ranges from 33% to 100%[88,89]. It is generally agreed that HBcAb-positive donor livers should be preferentially used in recipients who were HBV-positive prior to transplantation, since these patients require HBV treatment after operation[90]. For liver recipients unvaccinated and unexposed to HBV and who receive HBcAb-positive livers, prophylactic regimens remain to be defined. If they have never been exposed to any antiviral drugs, LAM and HBIG are regarded as the optimal prophylactic regiments. The infection rates of HBV with LAM alone or combined with HBIG treatments are approximately 10%[91,92]. In a 12-year study period, Chang *et al*[93] reported that LAM monoprophylaxis was effective in preventing *de novo* HBV in the majority of recipients, with only 8% of patients developing *de novo* HBV. Considering the high resistance rate of LAM, another study applied ADV monotherapy for liver transplant recipients who received HBcAb-positive livers. All recipients had undetectable HBV DNA after a 1.8 years[94]. The effectiveness of ETV and TDF in this context is still unknown. Recently, Ohno *et al*[95] evaluated whether active immunization prevented post-transplantation *de novo* HBV infection in patients who received HBcAb-positive liver grafts. They found that 90% of recipients acquired active immunity after four vaccinations. None had any side effects from HBV vaccination and none developed HBV infection during the study period. These results indicate that vaccination provides a new effective strategy for prevention of *de novo* HBV infection following liver transplantation in recipients who were given HBcAb-positive liver grafts. Furthermore, due to the shortage of donor liver and highly effective antiviral therapy, scientists started to use HBsAg positive donor, Loggi *et al*[96] reported 10 patients underwent liver transplant from HBsAg positive donors, for HBV-related disease (n = 6) or HBV-unrelated disease (n = 4). With sufficient antiviral therapy, HBV replication was effectively controlled. Besides, more encouraging outcomes of recipients who received HBsAg positive donors were also achieved by other centers[97,98]. These studies shed light on the new era of liver transplant with HBsAg positive donors.

Prophylaxis for de novo HBV following liver transplantation

Although effective antiviral therapy enables the wide use of HBcAb-positive donors, many unanswered questions remain regarding the risk of *de novo* HBV and the appropriate prophylactic measures that should be taken. It should also be noted that the degree of risk also depends on the immune status of recipients[99]. In our pediatric liver transplantation center, we found that intrahepatic HBV DNA in allografts may be a risk factor for *de novo* HBV infection in pediatric recipients of HBcAb-positive allografts[100]. While the use of HBcAb-positive donors increases, it is of utmost importance to exercise best preventative practices. *De novo* HBV is caused by two main reasons. First, the incidence rate of *de novo* HBV is significantly higher in the recipients who did not receive any prophylactic therapy compared with those who were treated with NAs[101]. Second, *de novo* HBV infection occurs in recipients who develop antiviral drug resistance. For these patients, mutant surveillance and change of NAs are necessary[102]. HBcAb-positive donors can be used as a strategy to increase donor pool on the premise of effective antiviral prophylaxis with NAs and close surveillance. The strategies for preventing HBV recurrence for different groups of HBV-related liver transplant recipients are listed in Table 3.

CONCLUSION

Improvements in the effectiveness and management of antiviral NAs have substantially improved the outcomes of HBV-related liver transplantations. Optimal treatment varies from case to case, but drug resistance is a major consideration in choosing the best option. ETV and TDF are currently the treatment choice for patients with drug resistance, but they are typically combined with HBIG. Since HBIG is costly and inconvenient, HBIG-free regimens are actively explored. Recombinant HBV vaccination is also emerging as an effective strategy for preventing post-transplantation HBV recurrence. Lastly, the ability to use HBcAb-positive donors is an encouraging step to increase the supply of donor livers. Personalized treatment seems to be the key future strategy in the treatment of HBV-infected patients, but novel antiviral agents with a high barrier to resistance should also be pursued to minimize drug resistance and to treat and prevent HBV prevalence.

Table 3 Strategies for preventing hepatitis B virus recurrence for different groups of hepatitis B virus-related liver transplant recipients

Low risk recipient (undetectable HBV DNA before transplantation)	NAs + HBIG[52] NAs + short-term HBIG, then switch to NAs combined therapy[58,59] or NA monotherapy (ETV or TDF)[66] NAs combined therapy[54,68] NA monotherapy (ETV or TDF)[69] (Further studies are needed)
High risk recipient (detectable HBV DNA before transplantation or with limited antiviral options)	NA + long-term HBIG[56]
Recipient with HBcAb positive donors	NA + HBIG[91,92] NA monotherapy[93,94] Active immunization[95]

ADV: Adefovir dipivoxil; ETV: Entecavir; HBcAb: Hepatitis B virus core antibody; HBIG: Hepatitis B immune globulin; HBV: Hepatitis B virus; LAM: Lamivudine; NA: Nucleoside analogue; TDF: Tenofovir.

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REVIEW

Hepatitis C virus infection: Are there still specific problems with genotype 3?

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Abstract

Hepatitis C virus (HCV) infection is one of the most common causes of chronic liver disease and the main indication for liver transplantation worldwide. As promising specific treatments have been introduced for genotype 1, clinicians and researchers are now focusing on patients infected by non-genotype 1 HCV, particularly genotype 3. Indeed, in the golden era of direct-acting antiviral drugs, genotype 3 infections are no longer considered as easy to treat and are associated with higher risk of developing severe liver injuries, such as cirrhosis and hepatocellular carcinoma. Moreover, HCV genotype 3 accounts for 40% of all HCV infections in Asia and is the most frequent genotype among HCV-positive injecting drug users in several countries. Here, we review recent data on HCV genotype 3 infection/treatment, including clinical aspects and the underlying genotype-specific molecular mechanisms.

Key words: Hepatitis C; Genotype 3; Direct-acting antivirals; Interferon; Hepatocellular carcinoma

Core tip: This article reviews the complex relationship between hepatitis C virus (HCV) genotypes and the possible complications in chronically infected patients. We discuss recent updates on the epidemiology and clinical aspects of HCV genotype 3 infection, including the currently available therapies. We also describe model systems to study the HCV genotype-specific molecular mechanisms.

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INTRODUCTION

Currently, about 3% of the world population is infected by the hepatotropic virus responsible for hepatitis C[1]. Thanks to intense research during the last two decades, hepatitis C virus (HCV) life cycle is now well known[2-4]. HCV is a small enveloped virus belonging to the *Flaviviridae* family and the hepacivirus genus, with a plus-strand RNA genome of about 9.6 kb. The HCV genome consists of a single open reading frame that encodes a large polyprotein of approximately 3000 amino acids. This polyprotein is processed by host and viral proteases to generate three structural (core, E1, E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B)[5]. Due to the error-rate of its RNA-dependent RNA polymerase NS5B, the high levels of viral replication and the pressure exerted by the host immune response, HCV sequence is highly variable, resulting in its classification in seven genotypes (GT) and 67 subtypes[6].

Two recent studies estimated the global burden of HCV infection and genotype distribution[7,8]. GT-1 is the most prevalent worldwide (46%), followed by GT-3 (22%). HCV genotype distribution shows geographic variations that reflect transmission mode differences and ethnic variability. Genotype diversity also directly affects the infected patients' outcome due to genotype-specific differences in response to treatment and disease severity. Until recently, HCV GT-3 was considered to be an easy-to-treat infection by using the standard combination of pegylated interferon α (PEG-IFN) and ribavirin (RBV), with higher cure rates (about 70%) than the other viral genotypes (particularly GT-1)[9]. In 2011, the approval of the first HCV protease inhibitors, in combination with PEG-IFN and RBV, greatly improved the treatment of HCV GT-1 in Europe and the United States. However, due to the side effect profiles and costs per sustained virologic response (SVR), this triple combination is no longer recommended by the European Association for the Study of the Liver (EASL recommendations 2015[10]). Indeed, several more effective and better tolerated direct-acting antivirals (DAAs) are now in clinical development[11], or have been approved by the Food and Drug Administration and the European Medical Agency (EMA). Among them, a nucleotide analog inhibitor of the HCV RNA-dependent RNA polymerase (sofosbuvir), a second-generation protease inhibitor (simeprevir) and two HCV-NS5A inhibitors (daclatasvir and ledipasvir) can be used in combination therapies.

Despite the wide range of new DDAs, few therapeutic options are effective for HCV GT-3. Moreover, the high cost of the new treatments implies a careful patient selection and will limit treatment delivery in some regions of the world. Here, we discuss the specific features and current issues of HCV GT-3 infection/treatment, including clinical aspects and the underlying molecular mechanisms.

EPIDEMIOLOGY

Although persistent HCV infection is one of the leading causes of liver-related morbidity and mortality, possibly accounting for up to 0.5 million deaths every year[12], its epidemiology remains poorly understood in many countries. As the efficacy of current and new therapies differ according to the HCV genotype, epidemiological data on the infected populations and the HCV genotype distribution have important clinical implications. Several recent studies on the global, regional and national prevalence and genotype distribution of HCV infection highlighted significant geographical differences[7,13-15] (summarized in Table 1). Specifically, HCV GT-3 accounts for 40% of all HCV infections in Asia, with a high prevalence in India, Malaysia and Pakistan (54%, 59% and 79%, respectively)[8]. HCV GT-3 is also predominant (> 43%) in some European countries (Denmark, Finland and United Kingdom) and might represent 50% of all HCV infections in Norway[14] and about 36% in Australasia[8]. The genotype distribution in a country may change from one year to the other, partly due to the migration of infected individuals and therefore, needs to be regularly updated. For instance, a recent study conducted in the southern part of Turkey reported a HCV GT-3 prevalence of 46%, a rate remarkably higher than that from previous Turkish findings[16] (Table 1). Updated data from four regions of Thailand and from Southeast Asia also indicate variations in the distribution of HCV genotypes and subtypes, notably in the 3a/3b subtype ratio[17] (Table 1). Phylogenetic analysis of HCV subtype 3a, the prevalent subtype in Thailand, showed that the genotypes of HCV samples from infected Thai and Indian/Pakistani patients cluster in close proximity, supporting the hypothesis of a close relationship between the HCV subtype 3a viruses that circulate in these countries[18].

Over the last ten years, GT-3 was reported as the most frequent genotype among HCV-positive injecting drug users (IDUs) in several countries[19-21]. In 2011, Nelson *et al*[22] performed a global systematic review of HCV prevalence among IDUs and found that about 10 million IDUs worldwide might be HCV-positive. China, the United States and the Russian Federation have by far the largest HCV-positive IDU populations. Thus, IDUs are now at the heart of the HCV epidemics in developed countries[23,24]. More specifically, HCV subtype 3a, which originated from Asia, has spread widely among IDUs and also among other patient groups in industrialized countries[25]. Recently, a prospective, multicenter cohort study on the treatment of HCV/HIV-positive patients after liver transplantation in Spain indicated that co-infected patients were

Table 1 Reported prevalence of hepatitis C virus genotype 3

Country, Region	Viremic population	Genotype 3 prevalence	Ref.
Asia			
India	6026 (3157-7174)	54.4%	[8]
	8666 (5150-15449)	64.0%	[14]
Malaysia	237 (47-1216)	58.6%	[8]
Pakistan	7039 (1728-10524)	79.0%	[8]
Thailand	925 (633-1259)	44.2%	[8]
Thailand, North		23%/16% (3a/3b)	[17]
Thailand, South		38%/14% (3a/3b)	[17]
Europe, Western			
Denmark	21 (14-21)	43.0%	[8]
Finland	22 (16-27)	46.0%	[8,14]
Norway	29 (25-37)	28.1%	[8]
	22 (18-28)	50.0%	[14]
United Kingdom	210 (125-428)	43.8%	[8]
Middle East			
Turkey	434 (274-959)	4.9%	[8]
Turkey, province of Kahramanmaras		46.0%	[16]
Australasia			
Australia	234 (169-260)	36.8%	[8]
New Zealand	50 (27-72)	35.0%	[8,14]

younger and had more frequently HCV GT-3 than patients infected only by HCV[26].

CLINICAL COURSE

Pathologies frequently associated with HCV genotype 3

Most HCV infections are asymptomatic. Consequently, infected individuals are not aware of their illness until the appearance of severe and irreversible liver disease, often several decades after the initial infection. Steatosis, characterized by lipid droplet accumulation in the cytoplasm of hepatocytes, is an extremely common histological finding in patients with chronic HCV (from approximately 40% to 80%, depending on the studies) and its prevalence is two times higher than in the general population[27]. Several risk factors for steatosis and liver injuries in HCV infection (*i.e.*, high fat diet, chronic alcohol consumption, dyslipidemia, obesity, chronic drug consumption, diabetes, insulin resistance, *etc.*) are the same as for alcoholic and non-alcoholic steatohepatitis, thus rendering the precise relationship between steatosis and HCV difficult to determine[28,29]. Nevertheless, in 1997, a study on the liver histopathological lesions in 90 HCV-positive patients revealed a significantly higher prevalence of steatosis among patients infected by HCV GT-3a than by GT-1a or 1b[30]. Since then, several other works confirmed this association and provided evidence of a HCV GT-3-specific cytopathic effect[31,32]. In a later study, Rodriguez-Torres *et al*[33] reported that among 614 patients, those with HCV GT-3 infection were more likely to have steatosis than patients infected by HCV GT-2 (79% *vs* 59%). This HCV genotype was qualified as steatogenic. Indeed, several studies reported a significant improvement in steatosis in HCV GT-3 patients who achieved sustained viral clearance after antiviral therapy[34-36], Furthermore, GT-3 patients tend to have hypocholesterolemia and hypobetalipoproteinemia[37-39], thus accounting for a direct effect of the virus on lipid metabolism. HCV GT-3 infection also promotes liver fibrosis development/progression[40-43]. However, the coexistence of host and viral factors contributing to liver steatosis and fibrosis in the same patient impair the analysis of their independent involvement and this issue remains controversial[44,45].

Moreover, chronic HCV induces multiple defects in the upstream components of the insulin signaling pathway in the liver, thus contributing to the observed prevalence of insulin resistance (IR) and type 2 diabetes mellitus in infected patients[46-48]. A genotype-specific association between IR and HCV was recently confirmed in a study on 497 HCV GT-1-positive patients and 541 GT-2/3-positive patients who received IFN-based therapy and in whom IR was measured before and 12 wk after the treatment using the homeostasis model assessment of IR (HOMA-IR)[49]. SVR was associated with a reduction in the HOMA-IR value in HCV GT-1-positive patients, but not in those with HCV GT-2 or GT-3 infection. Of note, IR was identified as an independent predictor of advanced fibrosis in patients with chronic HCV GT-3 infection[45]. However, the mechanism of HCV-mediated IR and the genotype-specific association remains unclear.

Evidence that viral factors, such as the HCV genotype, may affect the risk of progression to cirrhosis or to hepatocellular carcinoma (HCC) is scarce. Larsen *et al*[50] investigated the risk factors for these severe liver diseases in HCV-infected drug abusers in France between 2001 and 2007 and showed that HCV GT-3 infection is associated with severe liver disease in drug abusers, independently of age, sex, duration of infection, alcohol consumption and co-infection with HIV. In 2011, Nkontchou *et al*[51] reported the association of HCV GT-3 infection and higher HCC incidence in patients with cirrhosis in France. A strong association between chronic HCV GT-3a infection and HCC was also found in Pakistan[52]. This finding was recently confirmed by the analysis of patients' data from the Veterans Affairs HCV clinical registry showing that the risks of cirrhosis and HCC were significantly higher in HCV GT-3- than in GT-1-infected patients[53].

Conversely, HCV GT-3 was not reported as a significant factor influencing post-liver transplantation hepatitis, contrary to HCV GT-1, although the genotype influence on HCV recurrence after liver transplantation remains controversial[54].

Extra-hepatic manifestations

Besides liver disease, HCV infection can also cause a variety of extra-hepatic problems (autoimmune and/or

Table 2 Factors associated with poor response to pegylated interferon α/RVB in hepatitis C virus GT-3- infected patients

Factors	Study design	Number of patients	Characteristics	Ref.	Comments
Viral factors					
High baseline viral load (> 8 × 10^5 UI/mL)	Prospective	426 (all GT-3)	223 patients with viral load > 8 × 10^5 UI/mL	[56]	Combined with non-RVR
High baseline viral load (> 6 × 10^5 UI/mL)	Retrospective	107 (all GT-3)	45 non-SVR/62 SVR	[71]	Combined with advanced fibrosis
High baseline quasi-species complexity and diversity	Retrospective	10 (all GT-3)		[58]	
Mutations in NS5A	Prospective	49 (all GT-3)	25 non-SVR/24 SVR	[62]	Significant mutations at positions 2309 (Ala to Ser) and 2326 (Gly to Ala)
Host factors					
Fibrosis/Cirrhosis	Prospective	91 (all GT-3)	17 cirrhotic/74 non-cirrhotic	[63]	Also associated with increased risk of post-treatment relapse
	Retrospective	604 (all GT-3)	145 cirrhotic/459 non-cirrhotic	[64]	Response not affected by ethnicity
	Retrospective	180	108 GT-3/72 GT-2	[66]	Lack of SVR associated with fibrosis and GT-3
	Retrospective	107 (all GT-3)	45 non-SVR/62 SVR	[71]	Combined with high baseline viral load
Steatosis	Prospective	224	182 GT-3/42 GT-2	[69]	Lower SVR in GT-3
	Retrospective	932	505 GT-3/427 GT-2	[70]	Associated with higher relapse rates in GT-3 patients who had RVR
Ethnicity	Retrospective	103	66 Caucasians/38 Asians	[74]	Poor response could reflect more advanced liver disease at baseline in Asian patients
	Retrospective	604 (all GT-3)	305 non-Asians/299 South Asians	[64]	No correlation between ethnicity and treatment relapse
IFNL3 gene polymorphisms	Retrospective	107 (all GT-3)	45 non-SVR/62 SVR	[71]	No correlation between IFNL3 polymorphisms and SVR
	Prospective	293 HCV RNA- positive	65.87% GT-3/32.08% GT-1	[78]	CC and TT alleles strongly associated with SVR in GT-3 patients
Intrahepatic ISG15 expression/IFNL4 gene polymorphisms	Retrospective	92	36 GT-3/56 GT-1	[79]	In GT-3, low ISG15 expression and good-responder IFNL4 genotype associated with high SVR rates

GT: Genotype; SVR: Sustained virologic response; RVR: Rapid virologic response; NS5A: Non-structural 5A protein; IFNL: Interferon lambda; ISG: Interferon-stimulated gene; HCV: Hepatitis C virus.

lymphoproliferative disorders as well as cardiovascular, renal, metabolic and central nervous system diseases) in up to 74% of patients[55]. To the best of our knowledge, there is no evidence of a significant association between extra-hepatic diseases and HCV genotype.

HCV GENOTYPE 3 AND RESPONSE TO THE STANDARD TREATMENT

High SVR rates are observed in HCV GT-3-infected patients who receive the standard-of-care treatment (PEG-IFN/RVB). Accordingly, this genotype has been considered as "easy to cure" and grouped with GT-2 in clinical studies. However, increasing evidence indicates that differently from GT-2, some patients infected by HCV GT-3 respond poorly. Several viral or host factors could be associated with this reduced response (summarized in Table 2).

Viral factors

The baseline viral load is critical for treatment outcome. Indeed, HCV GT-3-infected patients with high pre-treatment viral load (> 8 × 10^5 IU/mL) are unlikely to show an SVR[56]. Moreover, it is well known that the high degree of genetic diversity of the HCV genome is associated with viral sensitivity or resistance to IFN-based therapy[57]. Accordingly, high HCV quasi-species complexity/diversity might negatively influence the outcome of IFN-based therapy in patients with chronic HCV GT-3 infection[58]. Viral genetic polymorphisms, especially within the non-structural 5A protein (NS5A) regions, may also be involved in the response to PEG-IFN/RVB therapy[57,59-61]. Specifically, in a small cohort of 49 non-responder and responder HCV GT-3a-infected patients, Mansoor *et al*[62] identified NS5A mutations that allow predicting the response to treatment.

Host factors

Several studies suggest that liver fibrosis and cirrhosis have a negative effect on the treatment response in HCV GT-3-infected patients[63,64] and that they are associated with an increased risk of hepatitis relapse after treatment[63]. Recently, a multicenter, open-label, randomized trial (*n* = 136 patients) showed that patients infected by HCV GT-3 and with advanced fibrosis do not benefit from extended therapy (48 wk) with PEG-INF/RBV[65]. Moreover, in a small cohort

of 180 Canadian patients, Powis *et al*[66] found a significant interaction between cirrhosis and HCV GT-3 (*vs* GT-2), leading to poor antiviral response. However, this association was also reported for other genotypes[67].

Among the severe liver injuries linked to HCV, steatosis negatively influences the response to antiviral therapy[68], particularly in HCV GT-3-infected patients[69], and is an independent predictor of relapse after rapid virologic response in these patients, irrespectively of the viral load[70]. However, using the database of a large prospective clinical trial in patients with HCV GT-2 or GT-3 infection, Rodriguez-Torres *et al*[33] found that liver steatosis did not affect the viral response in patients treated with PEG-IFN/RBV for 16 or 24 wk. A major limitation of many studies on SVR predictors is that they evaluated HCV GT-2- and GT-3-infected patients together. Therefore, Marciano *et al*[71] performed a retrospective multicenter study on 107 HCV GT-3-infected patients in Argentina and showed that advanced fibrosis and high pre-treatment viral load were associated with poor response to PEG-IFN/RBV in the patients who did not achieve an SVR.

Several studies focused on ethnic features that may influence the efficacy of IFN-based therapy, mainly by comparing Asian and Caucasian patients[72,73]. In 2008, a small study found a lower SVR rate in South Asian patients infected by HCV GT-3 than in Caucasians[74]. The influence of Asian ethnicity on the response to therapy for HCV GT-3 infection appears to be a major issue because this HCV genotype accounts for 40% of all infections in Asia. However, this association remains controversial because a retrospective analysis of 604 patients infected by HCV GT-3 and undergoing therapy in four United Kingdom centers (where many patients originate from the Indian subcontinent) showed that the response to antiviral therapy was affected by age, cirrhosis and diabetes, but not by the patient ethnicity (South Asian *vs* Caucasian)[64].

Polymorphisms of the interleukin-28B gene (*IL28B*, also named *IFNL3* for IFN lambda 3) located on chromosome 19 may affect both the natural history of HCV infection and the patient response to IFN-based therapy[75,76]. A recent systematic meta-analysis revealed a weak correlation between treatment outcome and IFNL3 genotype in patients infected by HCV GT-3 or GT-2[77]. However, in most studies analyzed in this review, these two genotypes were included in the same subgroup, thus rendering difficult to draw conclusions exclusively for GT-3. The recent study by Marciano *et al*[71] on 107 HCV GT-3-infected patients in Argentina did not find any association between *IFNL3* polymorphisms and SVR, while a study on HCV GT-3 infected patients in India showed that two favorable single nucleotide polymorphisms (SNPs) (rs12979860 and rs8099917) are strongly associated with SVR[78]. Holmes *et al*[79] evaluated the association between *IFNL3* and *IFNL4* (a variant upstream of *IFNL3* identified as a new *IFNL* gene[80]) genotypes, intrahepatic expression of IFN-stimulated genes (ISGs) and PEG-IFN/RBV treatment outcome in HCV GT-1 and GT-3-infected patients. Interestingly, this retrospective analysis of 259 patients treated with PEG-IFN/RBV in a single large tertiary center in Australia between 2004 and 2011 clearly highlights fundamental differences in the host response to HCV GT-1 and GT-3 infection, with a central role for intrahepatic ISG15 expression, independently of the *IFNL4* genotype. The lowest ISG15 level was observed in HCV GT-3-infected patients with the good-responder *IFNL4* genotype and the highest SVR. Conversely, the highest ISG15 level was detected in HCV GT-1-infected patients with the poor-responder *IFNL4* genotype and the lowest SVR rates. Robinson *et al*[81] compared gene transcription profiles in liver biopsies from uninfected and HCV GT-1- or GT-3-infected patients and confirmed the reduced predictive value of the *IFNL* genotype for HCV GT-3. Moreover, investigation of the host-pathogen interactions that underlie the genotype-specific clinical outcomes of chronic HCV infection[82] showed elevated ISG transcription in peripheral blood mononuclear cells from HCV GT-1-, but not from GT-3-infected patients, thus confirming the genotype-specific host-pathogen interactions.

HCV GT-3 infection after liver transplantation

Until recently, the treatment outcome of recurrent HCV GT-3 infection after liver transplantation was not precisely known. Faisal *et al*[83] demonstrated that the efficacy of the PEG-IFN/RBV combination for recurrent HCV GT-3 infection after liver transplantation is high and comparable with that in non-transplanted patients.

To improve the virologic response to IFN therapy, some combinations with other drugs were assessed. For instance, a case report indicated that the association of PEG-IFN/RBV and silibinin (an antiviral drug) resulted in an SVR after 24 wk in a treatment-naive patient who was reinfected by HCV GT-3 after liver transplantation[84].

Other drugs have been tested in combination with PEG-IFN and RBV. A prospective study on 179 treatment-naive patients with chronic HCV GT-1 or GT-3 infection indicated that fluvastatin (a 3-hydroxyl-3-methylglutaryl-coenzyme A reductase inhibitor of the statin family) combined with PEG-IFN/RBV improves the SVR in naive patients chronically infected by HCV GT-1 and high viral load, but not in patients infected by HCV GT-3[85]. Similarly, no significant benefit of statin in HCV GT-3 infection was found in a study based on the analysis of the United States Department of Veterans Affairs patient database[86].

Finally, severe side effects have been reported during IFN therapy and are one of the most frequent causes of treatment discontinuation[87,88]. Moreover, in specific groups of patients, IFN-based regimens are contraindicated or not applicable or failed repeatedly.

Table 3 Overview of direct-acting antiviral-containing treatments for hepatitis C virus genotype-3-infected patients

Drug combination	Recommendations[1]	Duration	Ref.
IFN-containing treatment			
PEG-IFN/RBV and SOF	For treatment-experienced patients, with or without cirrhosis	12 wk	[97]
PEG-IFN/RBV and DCV	Not yet recommended	12 or 16 wk	[98]
IFN-free treatment			
SOF and RBV	Suboptimal in treatment-experienced cirrhotic patients	24 wk	[99,100]
SOF and DCV	For patients without cirrhosis	12 wk	[101]
SOF/DCV and RBV	For treatment-naive and treatment-experienced patients with cirrhosis	24 wk	[102]
SOF/ledipasvir (single-tablet regimen)	Not yet recommended	12 wk	[104]
Promising therapeutic option			
GS-5816/SOF ± RBV	Under evaluation	12 wk	[107,108]

[1]According to the EASL Clinical Practice Guidelines in April 2015[10].

Therefore, IFN-free regimens need to be developed/tested in these patients.

GENOTYPE 3 IN DAA GOLDEN ERA

Until 2013 and given the relative efficacy of the standard-of-care treatment (PEG-IFN and RBV for 24 wk) for the other genotypes, research and drug development focused mainly on HCV GT-1. Currently, the introduction of DAAs has revolutionized HCV GT-1 therapy, while few effective treatment options are available for patients infected by HCV GT-3 who do not achieve an SVR with IFN (particularly, patients with cirrhosis), or have medical contraindications to IFN. First-generation, first wave NS3/4A protease inhibitors (telaprevir and boceprevir) are not efficient against HCV GT-3 infection[11,89,90]. For instance, Foster *et al*[89] reported that telaprevir alone or with PEG-IFN and RBV reduces the plasma levels of HCV RNA in patients with chronic HCV GT-2, but not GT-3 infection. Similarly, second-wave protease inhibitors, such as simeprevir (TMC435), are active against several HCV genotypes, but not GT-3[91,92]. The second generation of NS3-4A protease inhibitors, such as MK-5172[93], was developed to overcome the major limitations of first-generation antiviral drugs (*i.e.,* low barriers to resistance, dosing, clinically challenging side-effects and lack of pan-genotype activity)[94]. However, these drugs also are not effective against HCV GT-3 infection.

Therefore, despite the wide range of potent antiviral drugs, therapies approved in the United States and Europe for the treatment of HCV GT-3 infection include only two pan-genotypic DAAs that target key proteins in HCV replication: the first-generation NS5A inhibitor daclatasvir (DCV)[95] and sofosbuvir (SOF), a nucleotide analogue targeting the NS5B polymerase[96]. Currently, SOF is considered to be the backbone for the treatment of HCV GT-2-4 infections. These IFN-free, all-oral treatments are attractive, especially to avoid the many IFN-induced adverse effects. Nevertheless, the treatment choice mainly depends on the liver fibrosis stage and on the previous therapies. In April 2015, the EASL Clinical Practice Guidelines[10] recommended only three treatment options with DAAs for HCV GT-3 infections, in addition to PEG-IFN/RBV, and two of them are IFN-free regimens (summarized in Table 3).

IFN-containing treatments

A combination of PEG-IFN once per week and daily RBV and SOF for 12 wk can be used. Indeed, a phase Ⅱ clinical trial on 47 HCV GT-2 or GT-3 patients showed that this regimen gives high SVR rates in treatment-experienced patients with HCV GT-3 infection, irrespective of their cirrhosis status, and is well tolerated[97]. Although not recommended yet by the EASL in May 2015, other combinations were recently tested in clinical trials. Notably, the treatment duration with PEG-IFN/RBV could be reduced to 12 or 16 wk when combined with DCV in HCV GT-3- infected patients[98].

IFN-free treatments

For patients for whom IFN is not an option (*i.e.,* who do not have an SVR with the 24-wk PEG-IFN/RBV treatment, and for patients with medical contraindications), only two IFN-free regimens could be efficient. Again, clear differences were highlighted when HCV GT-3 and GT-2-infected patients were compared. For instance, two randomized, phase Ⅲ clinical trials (FUSION and POSITRON)[99] reported the need to extend the duration of the SOF/RBV treatment to 16 wk for HCV GT-3 infection, particularly in cirrhotic or treatment-experienced patients, to obtain a high response rate. Conversely, 12 wk were sufficient to achieve an SVR in HCV GT-2-infected patients. In the VALENCE study[100], therapy with SOF/RBV was extended to 24 wk for HCV GT-3-infected patients (compared to 12 wk in HCV GT-2-infected patients), resulting in high SVR rates. However, this combination was suboptimal for treatment-experienced cirrhotic patients.

The second IFN-free regimen that gives satisfying results for GT-3 patients is the combination of SOF and DCV for 12 wk, but only in non-cirrhotic patients. Indeed, in the ALLY-3 phase Ⅲ clinical

trial in treatment-naive (n = 101) or treatment-experienced (n = 51) HCV GT-3 patients, an SVR was achieved in 96% of patients without cirrhosis after 12 wk of well-tolerated treatment, while only 58% of cirrhotic patients achieved an SVR[101]. Similar results were reported by a French multicenter study on the compassionate use of SOF/DCV ± RBV in patients with HCV GT-3[102]. A regimen with a single tablet that contains ledipasvir and SOF (the two first-generation NS5A inhibitors) was recently approved in the United States and Europe and represents a significant advance for HCV treatment[103]. However, published studies are lacking on its use for HCV GT-3 treatment. Based on the few available data[104] and on the low *in vitro* efficacy of ledipasvir on NS5A activity[105], this combination is not recommended yet by the EASL in May 2015[10]. Among the many combinations tested to date, Gane *et al*[106] recently reported results from a phase Ⅱ pilot study that assessed the safety and efficacy of RBV combined with grazoprevir and elbasvir (two potent NS3/4 and NSA5 inhibitors, respectively) in treatment-naive, non-cirrhotic patients with HCV GT-3 infection. This combination was poorly efficient in this population.

Overall, the available data suggest that subgroups of patients infected by HCV GT-3, particularly those with advanced liver fibrosis or a previous failure of IFN-based therapy, are more difficult to cure with the short IFN-free regimens available in 2015. As achieving an undetectable viral load is associated with decreased hepatic morbidity and mortality[53], it is critical to improve the treatment for these subgroups of patients. Among the currently evaluated DAAs that are active against GT-3, the pangenotypic NS5A inhibitor GS-5816, combined with SOF ± RBV is a promising therapeutic option[107,108].

GENOTYPE 3 AND STUDY MODELS

Following the description of the association between HCV GT-3 infection and lipid accumulation in the liver in clinical studies, much effort has been made to understand the HCV genotype-specific pathogenic mechanisms. Detailed discussions of the different proposed molecular mechanisms are reviewed elsewhere and highlight the difficulties to identify pathological hallmarks of HCV GT-3 infection both *in vitro* and *in vivo* models[109,110].

Several evidences of perturbations induced specifically by HCV GT-3 infection, particularly the deregulation of lipoprotein metabolism, were obtained by analyzing liver biopsies and plasma samples from chronically infected patients[111-114]. As liver damage may be multifactorial, the major challenge in clinical research studies is to carefully exclude patients with risk factors for liver steatosis other than HCV (*i.e.*, patients with obesity, significant alcohol intake, diabetes, ongoing intravenous drug addiction, or use of steatogenic drugs).

In the last decade, it was suggested that the viral capsid core protein has a central and genotype-specific role. However, this hypothesis remains controversial[109]. Indeed, our current understanding of HCV GT-3 effects is largely based on studies using the human hepatoma Huh7 cell line that expresses the HCV GT-1b or GT-3a core protein[114-116]. However, these cell models do not mimic the liver physiological reality and complexity, or the viral infection processes and the specific intrinsic functions of other HCV GT-3 proteins.

Since 2005, a unique cell culture system based on the HCV GT-2a clone JFH1 and chimeric constructs allows the efficient replication of HCV and the *de novo* production of viral particles *in vitro* (HCVcc)[117]. Recently, we demonstrated that in well-defined culture conditions, adult primary human hepatocytes (PHH), isolated from liver tissue, support the complete infection cycle of natural HCV from patients' serum samples[118]. By testing the *in vitro* infectivity of about 120 HCV-positive serum samples in PHH cultures, we identified the highly infectious HCV GT-3a strain S310. We then cloned the full-length consensus genomic sequence of S310 and constructed a sub-genomic replicon, thus providing a new tool to study HCV GT-3a replication in Huh-7 cells[119]. We also identified critical mutations in subgenomic replicons that strongly promote viral replication and that were subsequently introduced in the full-length S310 HCV RNA. Kim *et al*[120] then demonstrated that full-length S310 clones replicate efficiently and produce infectious viral particles (HCVcc/S310) in Huh-7 cells, thus providing an unique infectious HCV GT-3a cell system. Interestingly, differences in HCV core protein localization and lipid content were observed in S310 (GT-3a)-infected and JFH1 (GT-2a)-infected cells. Moreover, the protease inhibitor telaprevir is less effective against S310- than JFH1-infected cells, as reported in the clinic.

Therefore, HCVcc/S310 particles represent a promising tool to determine the precise pathogenesis of HCV GT-3 infection and to understand the reasons of the effectiveness variations of different antiviral drugs. Further work is now needed to combine this HCV GT-3 strain with the most relevant host cell systems, such as primary cells, mouse models[121] or systems derived from tissue engineering[122].

CONCLUSION

Better understanding chronic HCV infection and the determinants of the treatment outcome appears nowadays as a key challenge. During the last few years, clinical research investigated mainly the interactions between viral and host factors, especially in HCV GT-3 infection. This genotype is significantly associated with severe liver disease and low response to most of the currently approved DAAs. The association of HCV GT-3 infection with the highest risk

of cirrhosis and HCC underscores the medical need for safe and effective treatment options for patients infected by HCV with this genotype. Luckily, recent clinical and basic research studies have been focusing more on HCV GT-3, in order to develop new strategies for providing timely and effective care for this high-risk population. Among the antiviral drugs under evaluation, host-targeted antivirals, like cyclophilin inhibitors, may be of great interest within the next few years.

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REVIEW

Role of matrix metalloproteinases in cholestasis and hepatic ischemia/reperfusion injury: A review

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Abstract

Matrix metalloproteinases (MMPs) are a family of proteases using zinc-dependent catalysis to break down extracellular matrix (ECM) components, allowing cell movement and tissue reorganization. Like many other proteases, MMPs are produced as zymogens, an inactive form, which are activated after their release from cells. Hepatic ischemia/reperfusion (I/R) is associated with MMP activation and release, with profound effects on tissue integrity: their inappropriate, prolonged or excessive expression has harmful consequences for the liver. Kupffer cells and hepatic stellate cells can secrete MMPs though sinusoidal endothelial cells are a further source of MMPs. After liver transplantation, biliary complications are mainly attributable to cholangiocytes, which, compared with hepatocytes, are particularly susceptible to injury and ultimately a major cause of increased graft dysfunction and patient morbidity. This paper focuses on liver I/R injury and cholestasis and reviews factors and mechanisms involved in MMP activation together with synthetic compounds used in their regulation. In this respect, recent data have demonstrated that the role of MMPs during I/R may go beyond the mere destruction of the ECM and may be much more complex than previously thought. We thus discuss the role of MMPs as an important factor in cholestasis associated with I/R injury.

Key words: Matrix metalloproteinases; Liver; Ischemia/reperfusion; Cholestasis

Core tip: Induction of matrix metalloproteinases (MMPs) modulates the progression of liver damage such as ischemia/reperfusion (I/R) injury and acute allograft rejection. The high incidence of biliary complications, after liver transplantation, is due to a cascade of events leading to biliary lesions to which cholangiocytes are particularly susceptible. This paper, while focusing on liver I/R and cholestasis, reviews factors and

mechanisms implicated in MMP activation/regulation together with the role of MMPs in biliary complications following I/R injury. Recent data support the view that MMPs play a dual role, both good and bad, in liver I/R depending on the length of time after damage.

Palladini G, Ferrigno A, Richelmi P, Perlini S, Vairetti M. Role of matrix metalloproteinases in cholestasis and hepatic ischemia/reperfusion injury: A review. *World J Gastroenterol* 2015; 21(42): 12114-12124 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i42/12114.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i42.12114

MATRIX METALLOPROTEINASES AND LIVER

Liver fibrosis arises from chronic damage to the liver associated with the over-accumulation of extracellular matrix (ECM) proteins, a characteristic of most types of chronic hepatic diseases[1] including: cholestatic liver diseases; primary biliary cirrhosis and secondary biliary cirrhosis; hepatotoxic diseases such as hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease (ALD), and non-alcoholic steatohepatitis (NASH)[2].

Advanced liver fibrosis disrupts the liver's normal architecture; hepatocytes are replaced with abundant ECM causing hepatocellular dysfunction and portal hypertension. Hepatic stellate cells (HSCs) are a major fibrogenic cell type in the liver[3]. Following liver injury, HSCs undergo an activation process and change their phenotype from quiescent retinoid storing HSCs to collagen-producing and contractile myofibroblast-like cells[4]. Activated HSCs migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM and regulating ECM degradation.

While the classic liver injury paradigm asserts that HSCs produce, remodel and turn over abnormal ECMs of fibrosis via MMPs, a recent paper by Calabro *et al*[5] has shown that MMPs are also secreted by other intrahepatic cell populations including hepatocytes.

Major changes in both quantity and composition of ECMs[6] and excessive ECM remodeling arises from a balance between increased synthesis and decreased degradation[7]. One class of zinc and calcium-dependent endopeptidases - matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) - plays a major role in the ECM remodeling[8]. Analysis of human and experimental animal fibrotic liver demonstrates an increase in a number of MMPs with a wide activity spectrum. Like many other proteases, MMPs are produced by activation of zymogens, which are released from cells[9-11]. Several different kinds of MMP have been identified (Table 1). Most of them can act on different collagen types, fibronectin, laminin, elastin, proteoglycans, and surface molecules such as growth factors or selectins.

MMP activity is regulated at three levels: gene transcription; posttranslational activation of zymogens, and interactions of secreted MMPs with specific inhibitors TIMP[12]. However, specific MMP inhibitors do not simply block protease activity but, on the contrary, the role of TIMPs is to modulate MMP functioning. Different protease activation occurs as a response to liver injury[13]. Usually, while injured cells release proteases, healthy cells release TIMPs; put another way, inhibitors are secreted by the cells surrounding these producing proteases[14-17]. Thus, high level of TIMPs occur simultaneously to an increase in proteases; in other words, both proteases and inhibitors could be produced by the same cell type at the same time[13]. TIMP concentrations and MMP/TIMP ratios are critical in this respect: a high MMP/TIMP ratio activate MMPs, while a low MMP/TIMP ratio lead to the opposite effect[18].

The uncontrolled ECM remodeling plays a central role in pathological changes leading to fibrosis[1,7]. A change in quality and quantity of matrix proteins occurs during fibrogenesis, resulting in excessive accumulation of fibrous tissue and an increase in ECM density[19] (Figure 1).

Several animal models of liver fibrosis have been developed, each of these with its strengths and weaknesses[20]. Bile duct ligation (BDL) has been used as an experimental model for chronic liver injury because of its closeness to hepatocyte damage, hepatic stem cell activation and the liver fibrosis found in human cholestatic liver disease[21].

The present study reviews and discusses the published articles searched on PubMed, MEDLINE, Google Scholar, and Google databases using specific keywords to identify articles related to MMPs in cholestasis and I/R injury. These keywords were "liver" and "MMPs," "cholestasis" and "ischemia/reperfusion". The search included letters to the editor, case reports, review articles, original articles, and meeting presentations published in the English-language literature from January 2000 to February 2015.

MMPs AND LIVER I/R

During liver resection, transplantation and trauma a prolonged oxygen deficiency is observed; the following oxygen restoration always induces reperfusion injury. In particular, the sequence of events that occurs during I/R injury is represented by an early increase in oxidative stress, liver sinusoidal endothelial cell damage, Kupffer cell activation and further release of reactive oxygen species, all of which in turn leads to marked tissue damage and liver remodeling[22]. MMPs are enzymes primarily involved in connective tissue remodeling; their inappropriate, prolonged or excessive expression has harmful consequences[23]. I/R is associated with gene expression, activation and release of MMPs, which have profound effects

Table 1 Classification and characteristics of the main matrix metalloproteinases

MMP (class and number)	Name	Extracellular Matrix substrate
Collagenases		
MMP-1	Collagenase 1	Collagen Ⅰ, Ⅱ, Ⅲ, Ⅶ, Ⅷ and Ⅹ, gelatin, proteoglycans, tenascin, entactin
MMP-8	Collagenase 2	Collagen Ⅰ, Ⅱ, Ⅲ, Ⅴ, Ⅷ and Ⅹ, gelatin, aggrecan
MMP-13	Collagenase 3	Collagen Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅸ and Ⅹ, fibronectin, gelatin, tenascin, aggrecan, osteonectin
Gelatinases		
MMP-2	Gelatinase A	Collagen Ⅰ, Ⅳ, Ⅴ, Ⅶ, Ⅸ and Ⅹ, gelatin, proteoglycans, elastin, fibronectin, laminin, aggrecan, versican, osteonectin
MMP-9	Gelatinase B	Collagen Ⅳ, Ⅴ, Ⅶ, Ⅹ and ⅩⅣ, gelatin, proteoglycans, elastin, aggrecan, versican, osteonectin
Stromelysins		
MMP-3	Stromelysins 1	Collagen Ⅲ, Ⅳ, Ⅴ, and Ⅸ, gelatin, proteoglycans, tenascin, fibronectin, laminin, aggrecan, versican, osteonectin
MMP-10	Stromelysins 2	Collagen Ⅲ, Ⅳ and Ⅴ, gelatin, proteoglycans, aggrecan, elastin, casein
MMP-11	Stromelysins 3	Collagen Ⅳ, fibronectin, laminin, gelatin, transferrin
Membrane type		
MMP-14	MT1-MMP	Collagen Ⅰ, Ⅱ and Ⅲ, fibronectin, vitronectin, tenascin, laminin, proteoglycans, aggrecan, elastin, casein, entactin
MMP-15	MT2-MMP	Fibronectin, tenascin, laminin
MMP-16	MT3-MMP	Collagen Ⅲ, fibronectin, casein, gelatin
MMP-17	MT4-MMP	ND
MMP-24	MT5-MMP	Activator of proMMP-2
MMP-25	MT6-MMP	Collagen Ⅳ, fibronectin, gelatin, fibrinogen
Others		
MMP-7		Collagen Ⅳ and Ⅹ, gelatin, proteoglycans, tenascin, fibronectin, laminin, aggrecan, osteonectin, entactin, casein, tranferrin, integrin b4
MMP-12		Collagen Ⅳ, gelatin, proteoglycans, fibronectin, laminin, entactin, casein, vibronectin, elastin
MMP-19	ND	Aggrecan, cartilage oligomeric matrix protein (COMP)
MMP-20	Enamelysin	Amelogenin
MMP-23A	MMP-21	ND
MMP-23B	MMP-22	ND
MMP-26	Matrilysin 2	Collagen Ⅳ, fibronectin, casein, fibrinogen
MMP-27	ND	ND
MMP-28	Epilysin	Casein

ND: Not Determined; MMP: Matrix metalloproteinase.

on tissue integrity[22,24] (Figure 2). A main role seems to be played by MMP-2 (gelatinase A; EC 3.4.24.24) and MMP-9 (gelatinase B; EC 3.4.24.35). These two gelatinases are the two main components of the space of Disse[25] and they are critically involved in the degradation of collagen Ⅳ and fibronectin[26]. Hence, increased activity of these MMPs may cause liver injury, with alterations of the sinusoidal cells and remodeling of the stromal structure. Already in 1997, Upadhya *et al*[27] demonstrated that MMP content, following release of MMP-2 and MMP-9 during cold preservation using rat and human liver perfusates, was dependent on the length of cold storage. Other data have since confirmed and extended the role of MMPs in hepatocyte cell death after prolonged cold storage and subsequent reperfusion[28]: the protective effects obtained using MMP inhibitors led the authors to suggest their addition to the liver preservation solution[28] (Table 2).

I/R injury is also typical of other pathological conditions in which the ischemic phase takes place under normothermic conditions. In particular, increased liver MMP-9 expression has been reported after normothermic I/R injury[29]. Moreover, serum MMP-9 has been found to be associated with the progression of liver damage in I/R injury[30], acute allograft rejection[31] and chronic viral hepatitis[32]. Some reports have suggested that specific MMP inhibitors decrease liver injury after normothermic ischemia associated with a concomitant reduction in inflammatory cytokine release[24,33] (Table 2). Other evidence has suggested that targeting MMP-9, using an anti-MMP neutralizing monoclonal antibody, leads to protection against damage after warm liver I/R; this approach appears to be more effective than using MMP inhibitors[23] (Table 2). Furthermore, experimental data suggest that MMP inhibition might be a promising approach in the context of pharmacological strategies designed to limit post-ischemic hepatic damage both in whole liver transplantation and in acute "small-for-size" liver graft injury[34].

That MMPs are secreted by Kupffer cells and hepatic stellate cells (HSCs) is a well-established fact[35]. MMP-9 are predominantly expressed in Kupffer cells, MMP-2 in HSC while MMP-3 and MMP-10 in hepatic stellate cells as well. Membrane type-1 MMP is found in significant amount in all liver cells.

However, another source of MMPs in the rat liver are sinusoidal the endothelial cells (SECs)[36]. In particular, the ability of HSCs to produce significant amounts of matrix degrading enzymes and their inhibitors has been demonstrated by Knittel *et al*[37]. In addition,

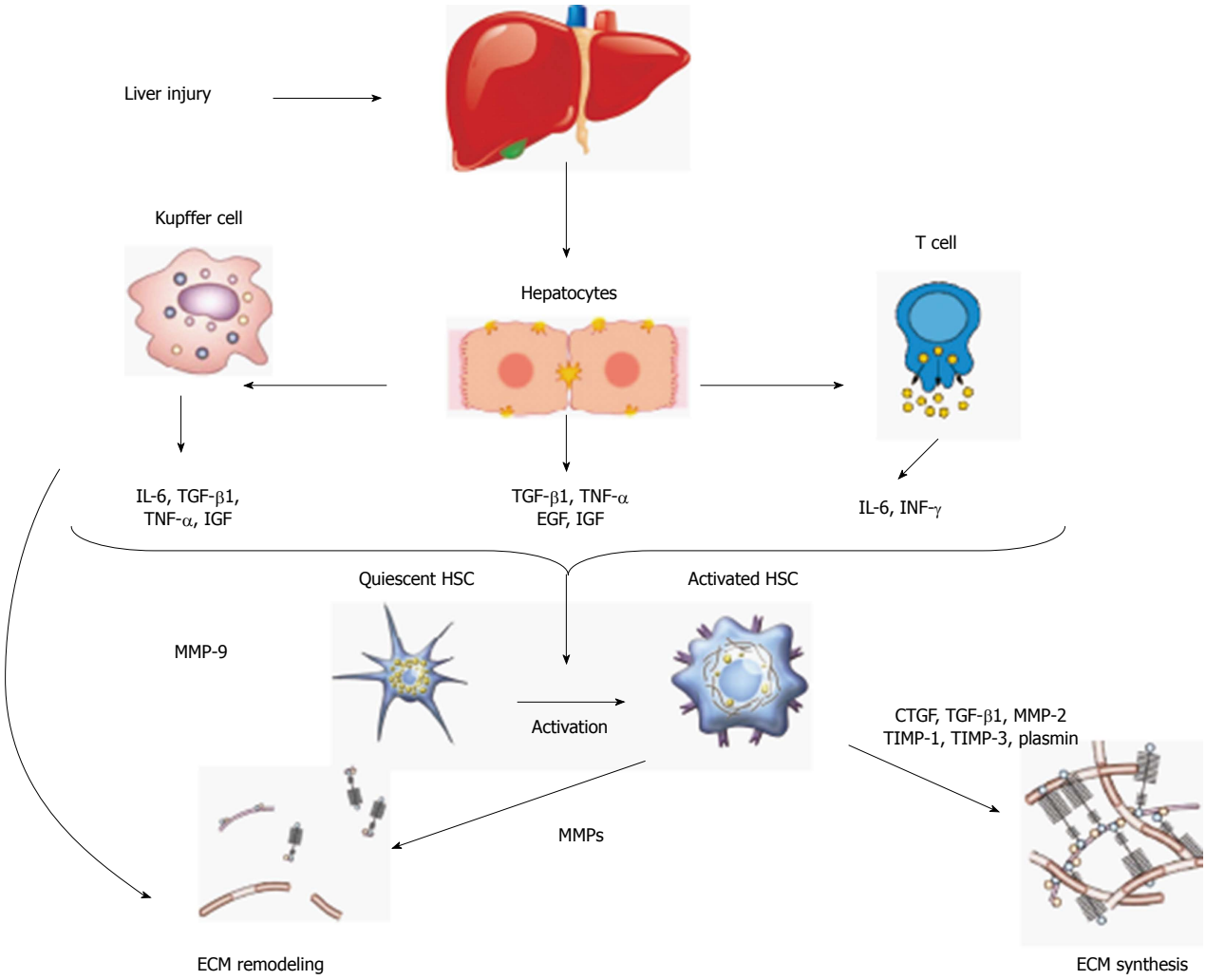

Figure 1 Mechanisms of hepatic fibrogenesis. IL-6: Interleukin-6; INF-γ: Interferon-γ; TGF-β1: Transforming growth factor-β1; EGF: Epidermal growth factor; IGF: Insulin-like growth factor; TNF-α: Tumor necrosis factor-α; HSC: Hepatic stellate cell; CTGF: Connective tissue growth factor; TIMP-1: Tissue inhibitor of metalloproteinase Type1; TIMP-3: Tissue inhibitor of metalloproteinase Type3; MMPs: Matrix metalloproteinases; ECM: Extracellular matrix.

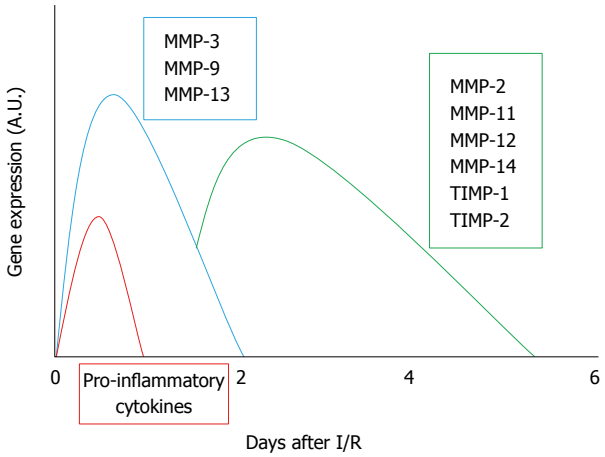

Figure 2 Time-course of matrix metalloproteinase expression following ischemia/reperfusion injury. In response to injury, pro-inflammatory cytokines, which promptly increase, induce expression of matrix metalloproteinases (MMPs) expression by hepatic cells including hepatic stellate cells (HSCs). The MMPs secreted by HSCs degrade the normal extracellular matrix (ECM) in the space of Disse.

hepatic MMPs, released from isolated rat SECs after preservation in cold Euro-Collins and UW solutions, increase as the length of time associated with cold preservation increases[27]. Hamada *et al*[23] have shown that MMP-9 expressed by leukocytes is also a key factor in cell transmigration and activation leading to liver injury. MMP-2 and MMP-9 are expressed not just in nonparenchymal liver cells but also in different subsets of leukocytes (T cells, neutrophils, monocytes, macrophages)[26].

Interestingly, recent data have demonstrated that the role of MMPs during I/R might be more complex than the mere destruction of the ECM or leucocyte recruitment to hepatic parenchyma[38]: the results have demonstrated that, although liver injury decreases in MMP-9-/- mice at 24 h after reperfusion, liver recovery after 72 h of reperfusion was significantly delayed in MMP-9-/- mice when compared with WT mice[38,39]. Thus, MMP-9 seems to play a dual role in liver I/R injury that varies with reperfusion times.

Table 2 Synthetic compounds involved in matrix metalloproteinase regulation

Compounds	Mechanism involved	MMPs involved	I/R model	Ref.
Bortezomib	Downregulation of pro-inflammatory (IL-1β, TNF-α and IFN-γ) and pro-fibrotic (VEGF, TGF-β, HGF, bFGF) factors	MMP-2 MMP-9	Steatotic orthotopic liver transplant	Tiriveedhi *et al*[45], *Transpl Immunol* 2014
KMUP-1 (NO-donor)	Protects from apoptosis-associated free radical generation and pro-inflammation	MMP-9	Hypoxic HepG2 cells	Kuo *et al*[90], *Int J Imm Pharm* 2013
CS-1 peptide	Blocks fibronectin α4β1 and decreases the release of pro-inflammatory mediators	MMP-9 MT-1-MMP/ MMP-14	Cold ischemia	Duarte *et al*[91], *Am J Transpl* 2012
CTT peptide	Reduction in TNF-α, IL-1, IL-2 and IFN-γ	MMP-9	Acute small-for-size liver graft	Ma *et al*[34], *Am J Transpl* 2010
Cyclic RGD peptide	Depresses inflammatory mediators (IFN-γ)	MMP-9	Steatotic Cold ischemia	Fondevila *et al*[92], *Am J Transpl* 2009
ONO-1714	iNOS inhibitor	MMP-9	Warm ischemia	Hamada *et al*[46], *Am J Pathol* 2009
RXP409	Inhibitory effects on MMP activity	MMPs	Cold ischemia	Defamie *et al*[28], *Hepatology* 2008
Anti-MMP-9	Decrease in expression of TNF-α, IL-2 and IFN-γ	MMP-9	Warm ischemia	Hamada *et al*[23], *Hepatology* 2008
CS-1 peptide	Blocks FN α4β1 integrin and its FN ligand	MMP-9	Steatotic orthotopic liver transplant	Moore *et al*[29], *Am J Pathol* 2007
ONO-4817	Reduction in TNF-α, IL-1β	MMP-2 and MMP-9	Warm ischemia	Shirahane *et al*[33], *Surgery* 2006
NAC	Reduction in free radicals	MMP-9	Warm ischemia	Chen *et al*[93], *Transpl Proc* 2005
BB3103	Prostaglandin PGE(1) protection	MMP-2	Cold ischemia	Yang *et al*[94], *Microvasc Res* 2002
RXPO3	Protects from necrosis/apoptosis	MMP-3-9-11-13	Warm ischemia	Cursio *et al*[24], *FASEB J* 2002

MMP: Matrix metalloproteinase; CS-1: Connecting segment-1; FN: Fibronectin; TNF-α: Tumor necrosis factor; TGF-β: Transforming growth factor β; IFN: Interferon; iNOS: Inducible nitric oxide synthase; IL: Interleukin.

PRO-INFLAMMATION MEDIATORS AND MMPs IN I/R

MMP expression and activity in liver I/R injury are topics under continuous development as are the factors involved in their activation. The mechanisms of I/R-induced liver injury include sequestration of inflammatory cells in the liver which causes oxygen radicals, nitric oxide (NO) and TNF-α to rise sharply[40]. In particular, research into the activation of Kupffer cells in I/R injury, which induces the production of proinflammatory cytokines including TNF-α and interleukin-1β (IL-1β), has led to an elucidation of the regulatory activity of cytokines on MMP expression and further suggested distinct roles for TNF-α and TGF-β1[41,42]; the early matrix degradation following liver damage may be enhanced by TNF-α, whereas the reduced matrix degradation observed during chronic tissue injury may be due to the TIMP-mediated action of TGF-β1 (Table 3). We recently demonstrated that the release of TNF-α, which occurs during the early stage of reperfusion after partial hepatic I/R injury, is related to an increase of MMP activity both in the ischemic region and in the non-ischemic lobe[43]. Furthermore, the increase in serum TNF-α after hepatic I/R is also correlated with MMP activation in the lung, a distant organ[44].

Other evidence has also shown that MMP expression by HSCs is regulated in a cytokine-specific pattern. Since TNF-α causes a marked stimulation of MMPs, it may well be that TNF-α and HSC are involved in initial matrix breakdown after liver injury. This initial matrix breakdown may be essential for early tissue repair reactions triggered by tissue inflammation when acute hepatic damage occurs[42]. Moreover, other data suggest that inflammatory cytokines such as TNF-α have a role in ECM degradation after liver I/R injury and that hepatic TNF-α expression runs parallel to MMP induction[26].

Recently, using an orthotropic liver transplant model in Zucker-obese rats, the administration of the proteosomal inhibitor, bortezomib, was shown to inhibit MMP activation and reduce serum proinflammatory cytokines including TNF-α and IL-1β[45] (Table 2).

Significantly, some experiments have also been performed to test the role of inducible nitric oxide synthase (iNOS) expression on the modulation of MMP-9 activity in hepatic I/R injury. Using both mice lacking the gene encoding for iNOS and mice treated with a selective iNOS inhibitor, the authors concluded that MMP-9 activity was induced by iNOS-derived NO and that this also led to detachment of hepatocytes from the ECM and cell death, in addition to increasing leukocyte migration through ECM barriers[46] (Table 3).

Fibronectin (FN) is involved in leukocyte adhesion, migration and activation. Amersi *et al*[47] reported

Table 3 Endogenous factors associated with matrix metalloproteinase modulation

Factors	MMPs Involved	I/R Model	Ref.
FN-α-β1	MMP-9 MT1-MMP/MMP-14	Cold ischemia	Duarte *et al*[91], *Am J Transpl* 2012 Coito[48], *Curr Opin Organ Transplant* 2011 Moore *et al*[29], *Am J Pathol* 2007 Fondevilla *et al*[95], *Transplant Proc* 2005 Amersi *et al*[47], *Am J Pathol* 2003
Tenascin-C	MMP-9	Warm ischemia	Kuriyama *et al*[96], *Hepatology* 2011
iNOS	MMP-9	Warm ischemia	Hamada *et al*[46], *Am J Pathol* 2009
TNF-α	MMP-2 and MMP-9	Warm ischemia	Feng *et al*[39], *J Surg Res* 2013 Palladini *et al*[43], *Toxicol and Pathol* 2012 Khandoga *et al*[26], *J Leukoc Biol* 2006 Chen *et al*[93], *Transplant Proc* 2005
IL-1β	MMP-2 and MMP-9	Warm ischemia	Shirahane *et al*[33], *Surgery* 2006
IL-6	MMP-9	Warm ischemia	Hamada *et al*[46], *Am J Pathol* 2009
IFNα-2a	MMP-2 and MMP-9	Cholestasis	Bueno *et al*[54], *J Hepatol* 2000
CD62	MMP-2 and MMP-9	Warm ischemia	Khandoga *et al*[26], *J Leukoc Biol* 2006
Plasmin	MMP-9	Cholestasis	Martínez-Rizo *et al*[97], *Liver Int* 2010
TGF-β	MMP-9	Warm ischemia	Feng *et al*[39], *J Surg Res* 2013
	MMP-13	Cholestasis	Aldaba-Muruato *et al*[98], *Can J Physiol Pharmacol* 2012
IL-10	MMP-2 and MMP-9	Warm ischemia	Feng *et al*[99], *Int Immunopharmacol* 2012

MMP: Matrix metalloproteinase; TNF-α: Tumor necrosis factor; iNOS: Inducible nitric oxide synthase; IL: Interleukin; IFNα-2a: Interferon α- 2a; TGF-β: Transforming growth factor β; FN: Fibronectin.

that blocking the interaction between FN and the integrin α4β1, the integrin receptors expressed on leukocytes, led to improved liver function in steatotic liver transplantation. Based on this evidence, they demonstrated that this is linked to a reduction in MMP-9 expression/activation on leukocytes of steatotic liver grafts[29]. MMP-9 expression during hepatic I/R was shown to be associated with massive leucocyte infiltration, extensive FN deposition and proinflammatory release, thus emerging as an important mediator of leukocyte traffic in liver I/R injury[48] (Table 3).

All this shows that numerous and rather complex mechanisms affect MMP modulation: for a list of endogenous compounds involved in MMP regulation see Table 3.

CHOLESTASIS AND MMPs

Biliary obstruction leads to a cholestatic inflammatory and fibrogenic process. Current evidence indicates that MMPs are of central importance for cholestatis-induced fibrosis but only limited evidence is currently available on their precise cellular origin and regulation within the damaged liver. Some authors have shown that marked alterations in the expression of MMPs and their inhibitors take place within the first week after BDL[49,50]. Specifically, they found that the proteolytic activities of MMP-2 and MMP-9 increased 2 d after BDL, peaked at day 10, and remained high throughout the study period[49]. The increase in gelatinase activities was accompanied by an increase in TIMP mRNA transcripts while no corresponding increase in TIMP protein activity was detected. This appears to arise from the formation of TIMP/MMP complexes. These findings suggest that complex changes in the local MMP/TIMP balance may underlie the pathological mechanisms of BDL fibrosis.

More recent publications support the view that analysis of the MMP activation not just 1-2 wk after BDL but even a few days after occlusion has a crucial role to play[51]. Ferrigno *et al*[50] have reported a marked alteration in gelatinase activity after BDL showing that this increase takes place in the first few days after BDL mainly in the right lobe. They also observed an increase in MMP-2 and MMP-9 that occurs significantly in the right lobe, more than in the median lobe and left lobe.

Although liver fibrosis has long been considered irreversible, recent studies suggest potential reversibility of liver fibrosis once the pathological trigger is removed[52]. Studies in patients with chronic hepatitis successfully treated with antivirals suggest recover even in cirrhotic patients[53]. In experimental models, reversibility of liver fibrosis depends on the degree of pre-established fibrosis. In an experimental model of cholestasis-induced fibrosis, MMP activity was upregulated in bile duct ligated rats treated with IFNα-2a. Bile duct ligation, itself, promoted MMP activity in both liver tissue and NPCs (non parenchymal cells) isolated from the same tissue[54].

In an elegant study, Popov *et al*[55] have shown that macrophages upregulate MMPs and become fibrolytic effector cells on apoptotic cholangiocyte engulfment *in vitro*, suggesting that phagocytosis-associated MMP induction in macrophages contributes significantly to biliary fibrosis reversal. A relevant finding of this study is the description of the subset of MMPs differentially regulated at the peak of matrix remodeling and degradation. In their study, the study

of expression patterns during biliary fibrosis reversal *in vivo* suggested that MMPs, with the exception of MMP-2, that have a profibrogenic role[56], and MMP-13, that could be involved in removal of the fibrotic matrix.

PRO-INFLAMMATION MEDIATORS AND MMPs IN CHOLESTASIS

During cholestasis a marked increase in liver and serum bile acid levels occurs, leading to acute liver toxicity, bile duct cells proliferation, and fibrosis progressing to cirrhosis[57-59]. However, the molecular mechanisms of liver injury induced by obstructive cholestasis remain unclear. Previous research has suggested a predominant hypothesis: inflammatory cell-mediated liver necrosis, and not bile acid-induced apoptosis, may be directly involved in cholestatic liver damage[60]. However, a recent study[61] indicates that bile acid composition between humans and rodents is different and that mechanisms of cholestasis in humans are different from rodent models.

In humans, during obstructive cholestasis, bile leaking back into the parenchyma can cause direct bile acid-induced necrosis, which, through release of damage-associated molecular patterns can initiate an inflammatory response.

Neutrophil accumulation has been directly implicated in the pathogenesis of early cholestatic liver injury[62,63]. After obstruction of the bile duct, an intense increase in biliary ductal pressure is produced[64] and this is quickly followed by ECM changes[65].

The accumulation of toxic bile acids induces hepatocyte injury, in part by activating death receptors[66]. This event triggers a secondary phase in which infiltration of inflammatory cells, activation of Kupffer cells and transformation of stellate cells to activated myofibroblasts occur, along with a MMPs-induced remodeling of the ECM. This structural hepatic changes further promotes liver injury and enhances hepatocyte apoptosis[67].

An increase in myeloperoxidase activity[68] and the formation of intracellular chlorotyrosine adduct in hepatocytes[62,63] are associated with neutrophil accumulation after bile duct ligation. The neutrophil-derived hypochlorous acid can induce liver injury by intracellular oxidative stress[69], prevented by inhibition of NADPH oxidase that protects against neutrophil cytotoxicity[70,71]. Furthermore, Nox1 and Nox2, hepatic NADPH oxidases respectively located in hepatic stellate cells and Kupffer cells, participate to BDL-induced fibrosis[72,73], though their role to the early liver injury has not yet been defined. Yang *et al*[74] suggest that the neutrophil-mediated liver injury is induced by MMP-induced cleavage of osteopontin (OPN), acting as an early pro-inflammatory signal after BDL in mice. In the cleavage of OPN into its pro-inflammatory form, MMP-3 and MMP-7 have a prominent role[75]. Yang *et al*[74] also reported that BDL induces MMP-3 early in the liver and, in addition, MMP-2, -3 and -9 activities increase in bile. Thus, probably, MMP-3 and other MMPs released into bile, activate OPN as potent chemoattractant for neutrophils. It is well known that MMPs are also involved in the modulation of cytokine and chemokine activity. MMPs can both generate chemotactic gradients by activating chemokines and cytokines, and inactivate these pro-inflammatory mediators[76]. The obstruction of the bile duct, induces an increase in biliary duct pressure, injuring the biliary epithelial cells. OPN and MMPs are released into bile and MMPs activates OPN, producing the factors attracting neutrophils. The high pressure in the biliary system occurring in BDL, provokes ruptures in the Canals of Hering. This process results in infiltration of bile into the parenchyma[77] and is facilitated by the expression on hepatocytes of intercellular adhesion molecule-1 (ICAM-1), induced by bile acids (BAs) into the parenchyma.

BILIARY COMPLICATION DURING ISCHEMIA/REPERFUSION INJURY

The development of biliary complications after liver transplantation is a major clinical problem, due to its relatively high frequency, complications, morbidity and even mortality. The formation of strictures in the liver bile ducts is accompanied by tissue remodeling in which MMP-2 and MMP-9 are considered to play a key role in connective tissue remodeling processes in the liver. The mechanisms by which ischemia/reperfusion (I/R) lead to liver injury are complex and multifactorial; these events also involve profound changes in MMP expression[24]. Based on the above considerations, further evaluation of a possible link between MMP-2 and 9 gene polymorphisms and non-anastomotic biliary strictures after liver transplantation might help explain MMP involvement[78]. Ten Hove *et al*[78] have shown that MMP-2 polymorphism is significantly associated with biliary strictures: genetically determined reduced MMP-2 tissue remodeling contributes to the development of biliary complications.

Reperfusion of liver grafts after cold preservation is associated with diminished bile production both in clinical liver transplantation and experimental models. Indeed, biliary complications represent a major surgical problem with an incidence of up to 30% after liver transplantation[79-81]. Cholangiocytes play a substantial role in the damage caused by preservation in hypothermic conditions: compared to hepatocytes and Kupffer cells, they are particularly susceptible to injury, and, in particular, to injury induced by cold hypoxia[82]. Hence, biliary strictures that occur after transplantation often require endoscopic, radiological and surgical procedures[83-85] designed to avoid graft dysfunction and/or re-transplantation.

Post-transplant biliary complications are usually classified into two types: (1) anastomotic strictures and (2) non-anastomotic strictures. Anastomotic

strictures of the biliary tree are located where bile duct anastomosis occurred and are generally well treated by stent placement[86]. Their incidence is between 5% and 10%[82]. Non-anastomotic strictures may be either extrahepatic (Type Ⅰ) or intrahepatic (Type Ⅱ). Arising from hepatic artery thrombosis, stenosis or ischemic cholangiopathy, they account for 10%-25% of stricture complications after liver transplantation[82]. In addition, ischemic cholangiopathy seems to be associated with prolonged periods of cold ischemic storage, delayed arterization of the graft or transplant from a donor after cardiac death (DCD) indicating that I/R injuries play a key pathogenetic role[82].

Clinical evaluation of biliary complications after liver transplantation has shown that a storage time of over 10-12 h leads to biliary strictures and other complications in more than 25% of liver transplant recipients[87]: a retrospective review of liver transplant patients demonstrated that liver grafts procured from DCDs showed a higher re-transplantation rate due to ischemic tract biliary lesions combined with severe intrahepatic cholestasis[88]. A meta-analysis and meta-regression of outcomes including biliary complications in donation after cardiac death liver transplantation published in 2014 confirmed and extended the finding that an increase in biliary complications, graft loss and mortality occurs with DCD liver transplantation[89]. Nevertheless the use of these organs needs to be balanced against the risk of recipients dying while on the waiting list[89].

CONCLUSION

Data from humans and experimental models supports the view that MMPs play a crucial role as modulators of tissue development, remodeling and repair in response to infection, disease of injury. Currently, it has been evaluating whether MMPs merely have a structural role in matrix remodeling, or they also have a role in regulating access to signaling molecules. One of the most important findings in MMP biology has been the realization that extracellular proteolysis is not only a mechanism that destroys structure or information. Instead, various studies have demonstrated that MMPs can release growth factors from the ECM and cell surfaces, activating latent proteins and generating new bioactive molecules through proteolysis.

Reperfusion damage is dependent on the degree of injury in previous phases and involves complex mechanisms and mediators that are not as yet completely understood.

Changes in extracellular MMP activities already occur in the early phases of reperfusion and are coupled with morphological changes to hepatic tissue, the biliary tree included. Significantly, as recent data have clarified, the multifactorial mechanisms of MMP modulation are associated to a possible dual role for MMPs during I/R injury; hence, only a detailed time-course evaluation of events occurring during reperfusion will provide specific indications for appropriate pharmacological treatments.

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REVIEW

Acute-on-chronic liver failure: Pathogenesis, prognostic factors and management

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Abstract

Acute-on-chronic liver failure (ACLF) is increasingly recognized as a complex syndrome that is reversible in many cases. It is characterized by an acute deterioration of liver function in the background of a pre-existing chronic liver disease often associated with a high short-term mortality rate. Organ failure (OF) is always associated, and plays a key role in determining the course, and the outcome of the disease. The definition of ACLF remains controversial due to its overall ambiguity, with several disparate criteria among various associations dedicated to the study of liver diseases. Although the precise pathogenesis needs to be clarified, it appears that an altered host response to injury might be a contributing factor caused by immune dysfunction, ultimately leading to a pro-inflammatory status, and eventually to OF. The PIRO concept (Predisposition, Insult, Response and Organ Failure) has been proposed to better approach the underlying mechanisms. It is accepted that ACLF is a different and specific form of liver failure, where a precipitating event is always involved, even though it cannot always be ascertained. According to several studies, infections and active alcoholism often trigger ACLF. Viral hepatitis, gastrointestinal haemorrhage, or drug induced liver injury, which can also provoke the syndrome. This review mainly focuses on the physiopathology and prognostic aspects. We believe these features are essential to further understanding and providing the rationale for improveddisease management strategies.

Key words: Acute on-chronic liver failure; Immune dysfunction; Systemic inflammatory response; Hepatic encephalopathy; Hepatorenal syndrome; Acute decompensation of cirrhosis; Liver failure; Organ failure; Severity score; Chronic liver failure-sequential organ failure assessment

Core tip: Acute on-chronic liver failure is a newly recognized syndrome characterized by acute deterioration of a compensated or decompensated chronic liver

disease, leading to organ failure, and a mortality rate ⩾ 15% at 28-d. Pathogenesis involves an exaggerated systemic inflammatory response in the setting of immune dysregulation and oxidative stress. Alcohol is a frequent precipitating factor seen most commonly in the West, and untreated hepatitis B virus infection is more prevalently seen in the East. However, it must be noted, that specific precipitant factors cannot be established in up to the 40% of cases. Recent prospective work has generated data on definition, prevalence, precipitating factors and scoring systems. Treatment of precipitant factors, complications, organ failure support, and liver transplantation are the current therapeutic options.

Blasco-Algora S, Masegosa-Ataz J, Gutiérrez-García ML, Alonso-López S, Fernández-Rodríguez CM. Acute-on-chronic liver failure: Pathogenesis, prognostic factors and management. *World J Gastroenterol* 2015; 21(42): 12125-12140 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i42/12125.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i42.12125

INTRODUCTION

In recent years, a new clinical form of liver failure has been recognised. Traditionally there were two types of liver failure: Acute liver failure (ALF), a rapid deterioration of the liver function in the absence of pre-existing liver disease, in the setting of an acute hepatic insult and chronic liver failure (CLF), a progressive and slow deterioration over the course of pre-existing end-stage liver disease[1-4]. In 1995, a third type of liver failure was first described[5]: Acute-on-chronic liver failure (ACLF). This new entity is characterised by acute complications of compensated or even decompensated cirrhosis and is characterised by a high rate of organ/system failure(s), and a high short-term mortality rate (> 15% at 28-d). Over the last decade, many definitions have been proposed, based on expert's opinion rather than on evidence-based data. The heterogeneity of definitions illustrates the differences in underlying aetiologies of liver disease between Eastern and Western countries[6-9]. The Asian Pacific Association for the Study of the Liver (APASL) defines ACLF as an "Acute hepatic insult manifesting as jaundice and coagulopathy, complicated within 4 wk by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease"[10]. Whereas, the American Association for the Study of Liver Disease (AASLD)/as well as the European Association for the Study of the Liver (EASL) consensus defines it as: "Acute deterioration of pre-existing, chronic liver disease (CLD), usually related to a precipitating event and associated with increased mortality at 3 mo due to multi-system organ failure"[6,11]. Given the lack of consensus among researchers, a group of investigators from the EASL-Chronic Liver Failure (CLIF) Consortium, undertook a prospective multicenter study in patients with cirrhosis suffering from acute decompensation (AD). The study identified patients with cirrhosis at a high risk of short term mortality. The study also aimed to develop a definition of ACLF. This large study was called EASL-CLIF Acute-on-Chronic Liver Failure in Cirrhosis (CANONIC)[12]. Based on data analysis obtained from 1343 hospitalized patients with cirrhosis and AD, at 29 liver units in 8 European countries this study established diagnostic criteria for ACLF. This study also permitted to know prevalence, precipitating factors, pathogenic mechanism and the phenotypic features of patients with ACLF.

DIAGNOSTIC CRITERIA OF ACLF

In the CANONIC study, the overall prevalence of ACLF was 30.9%. The definition of the ACLF diagnostic criteria was based on the presence of the 3 key characteristics of the syndrome: (1) AD: defined by acute development of large volume ascites, hepatic encephalopathy (HE), gastrointestinal haemorrhage, bacterial infections, or a combination of any of these[4,13-16]. In other words, the acute development of at least one of these major complications of liver disease must be present; (2) organ Failure: defined by a modified SOFA scale (Sequential Organ Failure Assessment) the CLIF-SOFA scale that takes into account some specificities of cirrhosis (Table 1)[17-21]; and (3) short-term mortality (28-d) at least 15%[12].

According to these characteristics, patients admitted to the hospital for an AD can be classified into 4 groups (Table 2). However, the majority of the patients did not have ACLF (77.5%). The Figure 1 summarizes the mortality rate according to the ACLF subtype.

PATHOPHYSIOLOGY

PIRO concept (predisposition, injury, response and organ failure)

The PIRO model is a useful approach in understanding the clinical sequence of the ACLF. It also consists of a scoring system that classifies severity, estimates risk, stratification, and prognosis in critically ill patients.

Initially postulated in 1900, and later modeled by Marshall *et al*[22] and Levy *et al*[23] the PIRO score was designed originally to measure the clinical features and outcomes in sepsis. The PIRO concept arises from the comprehensive examination of ACLF as a severe liver dysfunction, linked to other organs failure, as a strong and characteristic response to an insult that might be identified as an aggression within an underlying CLD that predisposes the whole situation[22]. It is proposed that organ dysfunction is the most predictive item among the four PIRO factors as it predicts 28-d mortality and multiple organ dysfunction[24]. Taking into account the great capacity of this concept to summarize and breakdown the physiopathology of ACLF, it has been proposed to also explain the cascade

Table 1 Chronic liver failure-sequential organ failure assessment score

Organ failure	0	1	2	3	4
Liver (Tbil, mg/L)	< 1.2	⩾ 1.2 to < 2.0	⩾ 2.0 to < 6.0	⩾ 6.0 to < 12	⩾ 12.0
Kidney (cr, mg/dL)	< 1.2	⩾ 1.2 to < 2.0	⩾ 2.0 to < 3.5	⩾ 6.0 to < 12	⩾ 5.0
			Or use of renal replacement therapy		
Cerebral (HE grade)	No HE	I	II	III	IV
Coagulation (INR)	< 1.1	⩾ 1.1 to < 1.25	⩾ 1.25 to < 1.5	⩾ 1.5 to < 2.5	⩾ 2.5 or PLT ⩽ 20×10^9/L
Circulation (MAP, mmHg)	⩾ 70	< 70	DA ⩽ 5 or DOB or Terlipressin	DA > 5 or E ⩽ 0.1 or NE ⩽ 0.1	DA > 15 or E 0.1 or NE > 0.1
Lung PaO_2/FiO_2	> 400	> 300 to ⩽ 400	> 200 to ⩽ 300	> 100 to ⩽ 200	⩽ 100
Or SpO_2/FiO_2	> 512	> 357 to ⩽ 512	> 214 to ⩽ 357	> 89 to ⩽ 214	⩽ 89

This score is used to categorize patients into grades of ACLF. ACLF: Acute-on-chronic liver failure; CLIF: Chronic liver failure; SOFA: Sequential organ failure assessment; Tbil: Total bilirubin; cr: Serum creatinine; HE: Encephalopathy; INR: International normalized ratio; PLT: Plateletes; DA: Dopamine; DOB: Dobutamine; E: Epinephrine; NE: Norepinephrine; PaO_2: Partial pressure of arterial oxygen; FiO_2: Fraction of inspired oxygen; SpO_2: Pulse oximetry saturation. Data from Moreau *et al*[12].

Table 2 Grades of acute-on-chronic liver failure according to the number of organ failure and the type of organ

No.	ACLF
ACLF grade 1	Single- organ failure (coagulation, liver, circulation, lungs) in patients with sCr 1.5-1.9 mg/dL and/or grades 1-2 HE or braine failure with sCr range from 1.5-1.9 mg/dL
ACLF grade 2	Two organ failures
ACLF grade 3	Three or more organ failures

Data from the CANONIC study[12]. ACLF: Acute-on-chronic liver failure; OF: Organ failure.

of facts in this entity.

Predisposition

Almost any kind of CLD can be a main predisposing factor on its own. In the Western countries, alcoholic cirrhosis is the cause of 50%-70% of all predisposing liver diseases of ACLF, comparing to the 10%-30% caused by chronic viral infection. In the Eastern countries hepatitis B virus (HBV) accounts for 70%, and only 15% is related to alcohol[6,10]. Nevertheless, some widespread infections like simple steatosis are not included as an underlying factor, whereas non-alcoholic steatohepatitis is. Also, metabolic and cholestatic liver diseases constitute part of susceptibility of the ACLF. This status of chronic liver impairment predisposes not only to an altered pro-inflammatory situation based on elevated serum cytokines, but also to a dysfunction in cellular immune system, reticulo-endothelial, and impairment in the bacterial translocation defense system[25].

Insult

Similarly to sepsis syndrome, infection may play a major role in triggering the whole inflammatory response. In the Asian continent HBV reactivation is one of the principal causes of ACLF. Other hepatotropic viruses like virus C reactivation might also provoke this failure[26]. In India, superimposed hepatitis E has been described as a major precipitant of ACLF[27,28].

Bacterial, fungal or viral primary infection can lead to systemic inflammatory response syndrome (SIRS) that has the potential to cause acute liver failure. In the CANONIC study, the principal infections related to ACLF were spontaneous bacterial peritonitis (SBP) and pneumonia[12].

Among the non-infective precipitating events, alcoholic hepatitis is one of the most common causes[12]. In the CANONIC study, one of the main predisposing events of ACLF was active alcoholism during the previous 3 mo (about 25%). Other situations described as precipitating events were less frequent (about 8%), and included acute toxic hepatitis, major surgery, or TIPS insertion. Paracentesis without adequate albumin replacement, has been reported as well[12,25]. However, in 40% of cases an obvious precipitating event could not be identified[12].

Host response

Host response is probably the leading factor in determining the severity of the ACLF and its prognostic outcome. The extension and range of inflammatory activation may result in the development of SIRS, characterized by a strong pro-inflammatory status (despite of an impairment of immune response) that can lead to ALF, and dysfunction in other organs.

Role of inflamatory response: The host immune response and the inflammatory cascade take especially high importance in this syndrome. The similarity between the SIRS produced by sepsis, and ACLF suggests that both entities share common pathogenic mechanisms. In SIRS there is an activation of the immune system relating leukocytes, endothelial cells, monocyte/macrophages, cytokines, enzymes, chemotactic mediators, and adhesion molecules overproduction. In this state, hepatocytes are believed to result in sensitized tumor necrosis factor (TNF)-induced apoptosis[29].

Comparing septic patients to ACLF patients, Wasmuth *et al*[30] formulated the concept of "sepsis- like immune paralysis" based on a profoundly decreased production

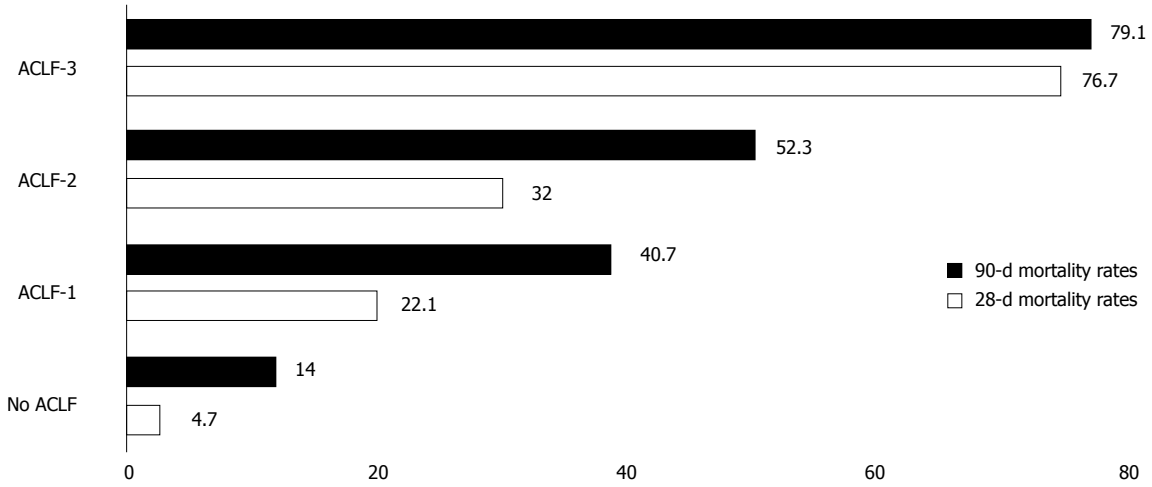

Figure 1 Mortality rates according to the grade of acute-on-chronic liver failure at 28 d and 90 d according to the grade of acute-on-chronic liver failure. Data from the CANONIC study[12]. Mortality increases with the grade of ACLF, directly related to the type and number of organ failure. ACLF: Acute-on-chronic liver failure.

of TNF-α and low monocyte HLA-DR expression in both groups. He postulated that this cellular immune impairment could contribute to increased mortality.

Endotoxins have also been proposed to play a role in mediating the full activation of neutrophils, which paradoxically would render them unable to act against the insult. An enhanced pro inflammatory cytokine environment was proved present in ACLF, as compared with cirrhosis alone. As ACLF is a pro-inflammatory state, it could result in chronically primed neutrophils, but in a deleterious form that might cause functional failure in phagocytosis due to a continuous energy depletion, which would prevent them from fighting against further infections[31].

The role that cytokines play in ACLF remains a key point in the pathogenesis of the inflammatory response. Elevated serum levels of many cytokines including TNF-α, sTNF-αR1, sTNF-αR2, interleukin (IL)-2, IL-2R, IL-4, IL-6, IL-8, IL-10, and interferon-α has been described. In particular IL-6 and TNF-α had been proposed to have a dual action, producing hepatocyte death and also enhancing hepatocyte proliferation through a complex interplay with Kupffer cells (KCs) and hepatocytes[10,32].

The mechanism in the rise of cytokines can be related to necrotic liver cells, decreased hepatic clearance or, probably the most important, activation of toll-like receptors (TLRs). These receptors activate KCs[33,34]. Causing KCs to change into M1 pro-inflammatory macrophages[35]. TLRs have the capacity to interact with many different agents, recognizing multiple molecular patterns in pathogen or damage- associated pathways[36]. KCs play a key role in liver injury, as they internalize ligands and activate the signaling cascades, transcription of pro-inflammatorycytokines, and superoxide agents. This promotes oxidative stress and releases proteolytic enzymes, vasoactive substances such as endothelin-1 (ET-1), thromboxane A_2, nitric oxide (NO), and prostaglandins, thereby contributing to microcirculatory dysfunction[37].

This entire cascade eventually leads to hepatocyte death and liver dysfunction. Hepatocyte apoptosis rather than necrosis seems to be the predominant mode of cell death in ACLF, as high levels of the apoptosis marker cytokeratin M30 occurs in ACLF patients[38]. Nevertheless, both paths are not mutually exclusive and the concept of "necroapoptosis" is only as of late been proposed. Also, the same patient can present both forms of cell damage dynamically[38].

Role of bacterial infection

Though the precise mechanisms involved in ACLF have yet to be clarified, the immune system seems to be play a predominant role in the setting of cirrhosis, which paradoxically is one of the most common forms of immunodeficiency[39,40].

The homeostatic role of the liver in the systemic immune response is well known[41-43]. This role is defines as "cirrhosis-associated immune dysfunction" which includes the main syndromic abnormalities of immune function, immunodeficiency, and systemic inflammation[44]. This is a dynamic condition which leads to oscillation from predominantly pro-inflammatory to predominantly immunodeficient situations[44,45].

Immune dysfunction in cirrhosis is multifactorial and reflects a complex interaction between many systems, predisposing these patients to infections. It is thought that this susceptibility is not due to a sole responsible factor, but rather to the concomitant presence of various facilitating mechanisms such as: portal hypertension with porto-systemic shunting (thus impairing detoxification and reticuloendothelial system phagocytic activity), increased gut permeability and bacterial overgrowth (all of them increases the risk of bacteremia and the occurrence of endotoxemia), albumin and lipoprotein dysfunction, or aberrant toll-like receptor expression in KCs[33,46-49].

The presence of innate immune dysfunction in ACLF can be inferred from susceptibility to infections: 30% to 50% of cirrhotic patients presented bacterial infections upon their admission or during hospitalization[50-53]. The most common bacterial infections are SBP (25%), urinary tract infections (20%), pneumonia (15%) and spontaneous bacteremia (12%)[54]. Clinical and biochemical parameters in bacterial infection were generally correlated with the severity of liver disease. Child-Pugh score (CPs) showed a predominance of class C in infected cirrhotic patients compared to non-infected ones[55].

PATHOPHYSIOLOGY OF ORGANS FAILURE

Hepato-adrenal axis failure

Adrenal dysfunction is frequently reported in patients with CLD (compensated or decompensated), and severe sepsis (51%-68%), especially in patients with high CPs, model of end stage liver disease (MELD) scores, and hemodynamic instability, thereby reflecting a more advanced liver disease[56-58]. Some hypotheses to explain adrenal dysfunction pathophysiology have been proposed, such as: decreased cholesterol levels, overstimulation of the hypothalamus-pituitary-adrenal axis by cytokines, and endotoxemia[56,59]. However, the mechanism leading to adrenal insufficiency remains unclear[60].

This dysfunction called "hepato-adrenal syndrome", is associated with renal failure, hemodynamic instability, and increased mortality. Hydrocortisone administration can have initial favourable effects on hemodynamic parameters, but it has not been confirmed to improve the outcome[57,61].

Test to assess adrenal function and its interpretation in cirrhosis and ACLF is difficult due to the absence of consensus, and normal values of this test, therefore recommendations cannot be made[62].

Pulmonary failure

Respiratory failure can be classified in two types of complications. First, complications typically related to cirrhosis, like hepatic hydrothorax (that can become infected), portopulmonary hypertension, hepatopulmonary syndrome, and transfusion-related acute lung injury (among others)[63,64]. Secondly, infectious complications (which are the most common), like aspiration pneumonia. Bacterial respiratory tract infections in cirrhotic patients represent 14% to 48% of all bacterial infections[65]. These patients are at increased risk of pneumonia due to unprotected airway from altered consciousness, increased intra-abdominal pressure from ascites, endoscopic procedures for gastrointestinal bleeding and increased risk of bacterial translocation because of excessive use of proton pump inhibitors[66-68].

The relevance of this OF, and its impact on mortality in ACLF, can be emphasized by its incorporation into the CLIF-SOFA score. Respiratory failure is defined in CLIF-SOFA by gasometric parameters, as a partial pressure of arterial oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ratio of 200 or less, or a pulse oximetry saturation/FiO_2 ratio of 200 or less[12,69].

Haematological failure

Failure in the coagulation system is defined in CLIF-SOFA as an international normalized ratio (INR) of 2.5 or more, or a platelet count < 20000/μL[12].

Patients with liver disease are in a state of "rebalanced haemostasis" which results in an increase of both pro-thrombotic and anti-thrombotic factors[70,71]. As explained above, in ACLF the inflammatory process may trigger the "unstable balance" to any of these two states and may be manifested by either bleeding or thrombotic complications. Anti-thrombotic alterations are thrombocytopenia, abnormal platelet functions, deficiency in the coagulation factors (except for Factor Ⅷ), and increased fibrinolysis. On the other side of the balance, the pro-thrombotic state, which is manifested by a decrease of anti-coagulation and plasminogen, associated with an increase in the plasminogen activator inhibitor (PAI), Von Willebrand factor and in factor Ⅷ[72-76]. Summarizing, the most significant haematological abnormalities described in ACLF are defective platelet function and increased fibrinolysis[77,78].

Coagulopathy is worsened in sepsis by the presence of endogenous low-molecular weight heparinoids which disappear with resolution of infection. In addition, there is an increased risk of bleeding complications due to further increased portal pressure secondary to infections and may explain the beneficial role of antibiotics administration in reducing early variceal rebleeding[79-81]. Standard laboratory values, such as the determination of the INR or the activated partial thromboplastin time, poorly reflect the pathophysiological changes in ACLF, therefore a deeper comprehension of underlying mechanisms is needed to guide correction of coagulation abnormalities on these patients[71,82].

Neurological failure

HE is a common manifestation of ACLF. Neurological failure is defined by CLIF-SOFA by the development of encephalopathy grade Ⅲ or Ⅳ[83]. Local and systemic disturbances have been implicated in the development of this syndrome. Patients with HE show a functional derangement in the blood brain barrier leading to increased transport of neutral amino acids and reduced transport of basic amino acids[84]. Elevated brain ammonia level and cerebral hemodynamic dysfunction are known to be the major etiological factors. Recent data suggest that in light of functional immunoparesis of patients with liver dysfunction, a poorly understood relationship between ammonia, inflammation and oxidative stress may underlie the HE pathogenesis[85-87]. These alterations include

abnormalities in neurotransmission [*i.e.,* disturbances in aminobutyric acid (GABA) ergic systems], energy impairment (*i.e.,* decrease in cerebral blood flow, inhibition of cerebral energy metabolism by ammonia), brain oedema (*i.e.,* elevated ammonia levels, hyponatremia) and neuro-inflammation (generation on nitric oxide, prostanoids, astrocytic swelling)[88,89].

EH in the setting of ACLF have a different course from cirrhotic patients with AD but without ACLF[90]. Isolated EH usually develops in the context of long-term diuretic use, and is not associated to an impairment of liver function. The absence of significant inflammatory reaction and the low prevalence of organ's failure relatively preserve good prognosis. By contrast, patients with HE associated with ACLF has an extremely poor survival rate, as a consequence of a generalized inflammatory reaction that may play a role in brain and other organs dysfunction. In addition to liver dysfunction, HE in the setting of ACLF is frequently associated with bacterial infections, active alcoholism or dilutional hyponatremia[91,92].

Circulatory failure

According to the CLIF-SOFA, patients requiring inotropic drugs are considered to present circulatory failure[6,12].

Mechanisms underlying haemodynamic and cardiac dysfunction in ACLF resembles closely to those in severe sepsis, as TNF and NO are increased and cortisol is decreased[57,58]. This circulatory dysfunction is typically characterised by an intense hyperdynamic state with the inability to obtain adequate perfusion pressure despite volume expansion and requirement of large doses of inotropic agents, with subsequent development of lactic acidosis[93-95]. The increased infections risk is often coupled to cardiac dysfunction. This situation may be aggravated by sepsis related to increased susceptibility to infections, by impairment in cardiac systolic and/or diastolic function or by the presence of hepatoadrenal syndrome[96,97]. It has been speculated that any acute inflammatory insult in patients with underlying cirrhotic cardiomyopathy may precipitate cardiovascular collapse[97]. In ACLF there is often incapacity to appropriately increase the cardiac output in response to the insult[98]. This finding is in contrast to decompensated cirrhosis, where cardiac output remains elevated, until advanced stages of liver disease, secondary to splanchnic vasodilatation. This cardiovascular abnormality is associated with an increased risk of death, particularly in those patients who present with renal dysfunction[99]. Inotropic support is often needed, however the best therapeutic approach remains unclear[100].

Kidney dysfunction

Renal failure is defined by the CLIF-SOFA as a creatinine $\geqslant$ 2 mg/d and the use of renal replacement therapy[12]. In the CANONIC study kidney failure was the most frequent OF for ACLF grades (55.8%), followed by liver, cerebral, and coagulation failures (43.6%, 27.7% and 24.1%, respectively)[12]. As shown, acute kidney injury (AKI) is a frequent and an important component of ACLF, as it is associated with poor prognosis[101-105]. Mortality is associated to the type and number of organs failure, and was higher in the subgroup of patients with single kidney failure than in those with involvement of other organs. A study including 562 hospitalised patients with cirrhosis, suggested that the most frequent causes of renal failure were related to bacterial infections (46%), hypovolaemia (32%), hepato-renal syndrome (HRS) (13%) and intrinsic renal failure (9%)[106]. Other studies support these results[107-109]. Renal failure may be categorized into four types: HRS, parenchymal disease, hypovolemia-induced and drug-induced renal failure[106,107,110]. Attributing the renal failure to a single mechanism in patients with multiorgan failure is usually difficult. There are many aetiologies of renal failure in patients with ACLF[111,112]. Prerenal factors are generally associated with renal hypoperfusion, which may be associated with intravascular volume depletion (haemorrhage, renal and gastro-intestinal fluid loss) or marked deterioration of effective arterial blood volume, leading to HRS[99]. Most intrarenal causes are related to ischemic acute tubular necrosis, also due to renal hypoperfusion[109,113].

Undoubtedly, systemic haemodynamics and cardiac dysfunction play an important role in the development of renal failure. Thus, in some patients the circulatory changes may predominate, whilst in other patients there may be increased synthesis of pro-inflammatory mediators (or both). SIRS has also been suggested to be involved, accompanying and aggravating the above mentioned mechanisms[114-116]. The benefit of the anti-inflammatory or immunomodulatory agents such as corticosteroids or pentoxifylline in the prevention of renal failure in patients with acute alcoholic hepatitis might support this observation[106,109,117,118].

Liver failure

Liver failure is defined by the CLIF-SOFA as a total bilirubin $\geqslant$ 12 mg/dL. The hallmark of the liver manifestation of ACLF is hyperbilirubinemia and coagulopathy[12].

Some lines of evidence suggest that the histopathological characteristics of the liver during ACLF will be determined by the underlying cause of cirrhosis and the nature of the precipitating event[6,25]. From the pathophysiological point of view, in ACLF there is a further exacerbation of haemodynamic derangements besides the already existing liver structural changes[6]. Liver inflammation has a capital importance on increased portal pressure[119]. Mechanisms proposed are changes in vascular smooth muscle cells, activation of hepatic stellate cells, reduced nitric oxide activity secondary to endothelial dysfunction and upregulation of sympathetic tone[113,120-123]. Another key component is angiogenesis, which plays an important role in

increasing intrahepatic resistance, and therefore in ACLF pathogenesis[124-127]. It should be noted that according to definition on diagnostic criteria, differences in portal haemodynamics have been described. When ACLF was defined according to the APASL criteria no differences were observed in portal haemodynamics between decompensated cirrhosis and ACLF[128]. However, using the EASL-AASLD definition, the portal pressure was markedly higher in those with ACLF portal pressure, in comparison to those with decompensated cirrhosis when ACLF was defined according to the AASLD/EASL definition[129]. These results point to the need for cautious definition of the population studied.

SIRS and bilirubinostasis had been associated with an increased risk of subsequent infection[130-132]. These infections begets a greater inflammatory response with aggravation in portal hypertension and further worsening of an already poor prognosis[80,133].This concept is supported by the reduction of portal pressure by antibiotics administration that modulates gut-derived endotoxemia and bacterial translocation[79,80,134].

Another characteristic feature of liver dysfunction is coagulopathy. Coagulation tests are usually abnormal in cirrhotic patients due to impaired synthesis and increased consumption of coagulation factors (see haematological failure). Bleeding abnormalities and hyper-coagulability may coexist[70,81,82,107].

PROGNOSIS, PREDICTORS OF MORTALITY

ACLF is associated with a high mortality rate of 50%-90% (which means it is 15 times higher of a rate in patients with ACLF), as compared to patients with an AD without ACLF[12]. Unfortunately, there are no well-established prognostic indicators available for predicting ACLF progression. The discrepancies and unevenness in the definition of ACLF, and therefore the different characteristics of the population under study, has limited research into the identification of clear indicators of severity and outcome predictors[9,135-138]. As previously mentioned, ACLF is a serious illness, in which reversibility is sometimes suggested in about half of the patients, or in other cases can progress to a life-threatening situation. It is, therefore, of fundamental importance to have accurate prognostic indicators in place, to be able to identify patients at high risk of ACLF that may require intensive care treatment, concise clinical decision making to improve management and minimize futile and expensive care. Due to a lack of universally accepted prognostic model for ACLF, many already widely used prognostic models for cirrhosis have been applied for the evaluation of this syndrome. In this regard prognosis scores can be categorized in two: the former that evaluates the severity of liver dysfunction (CPs, MELD) and the latter, global prognostic scores [Acute Physiology and Chronic Health Evaluation (APACHE Ⅱ) and SOFA]. Several lines of evidence demonstrate that global prognostic scores are superior to specific liver scores for estimation of prognosis in these patients[103,105,139-141]. These findings emphasize the importance of OF in defining the prognosis of ACLF, because once extrahepatic failure has begun, outcome is mainly determined by the degree of end-organ dysfunction and less by the severity of the liver disease[101,104,142-144].

Some studies suggested that APACHE-Ⅱ is the best predictive scoring system, owing to the fact that in ACLF once liver failure is established the prognosis is determined by the degree of other organ dysfunction and not by the severity of liver failure[10,98,142,145]. In some studies, MELD has been found to be a discrimination factor similar to SOFA and APACHE Ⅱ[146]. The CLIF-SOFA also proved to be a strong predictor of short-term mortality but does not significantly improve the prediction accuracy of MELD and MELD-Na[18,19]. Recently, based on data from the CANONIC study, a specific prognostic score for ACLF has been developed named the "CLIF-CONSORTIUM score for ACLF" (CLIF-C ACLF score). This score is the result of combining "CLIF-Consortium Organ Failure (CLIF-C OF)" score (designed for the diagnosis of ACLF), and two other independent predictors of mortality (age and white-cell count)[7,135,147]. This new score at ACLF diagnosis showed a significantly higher predictive accuracy than MELDs, MELD-Na and CPs[7,135]. CLIF-C ACLF score has also been shown to be an independent predictor of course severity[45,148].

Furthermore, ACLF has been shown to be dynamic process. In this connection, scoring taking into account dynamic changes, or improvement/impairment in the same score, have shown to predict outcomes[149,150]. In this line, Kumar *et al*[151] has demonstrated that any improvement in the MELD score over 2 wk suggests a good outcome.

A large number of studies have indicated that the greater the number of organ dysfunction or OF at diagnosis, the lower the ACLF patient survival[12,98,152,153]. The basic mechanism is the importance of systemic inflammation on OF, and its impact on prognosis[132]. Along these lines, ACLF mortality has been associated with loss of organ function (Higher CLIF-SOFA score), high leukocyte counts, and high C-reactive protein (CRP). ACLF is especially severe in patients with no prior history of AD, characterized by higher numbers of OF, higher levels of inflammatory mediators, leukocyte count and higher rates of mortality[12,154]. Patients with ACLF are younger than those without, and age is associated with more vigorous immune response[154]. These data sets do not coincide with the findings from Shi *et al*[155] suggesting that ACLF patients with or without prior decompensation had comparable short-term prognosis, but the former group was characterized by increased delayed mortality.

Table 3 Summary of predicting factors

High levels of bilirubin	Gustot *et al*[148], López-Velázquez *et al*[206], Cordoba *et al*[90]
Age and high INR	Shi *et al*[155], Cordoba *et al*[90], Garg *et al*[146], Kumar *et al*[151], Moreau *et al*[152]
Decreased serum thyroid-stimulation hormone (TSH) levels	Wu *et al*[207]
Low free T3 levels	Agiasotelli *et al*[208]
Systemic haemodynamic changes	Garg *et al*[146]
Iron metabolism and transport	Maras *et al*[209]
Neutrophil-lymphocyte ratio (NRL)	Lin *et al*[210], Liu *et al*[211]
Presence of SIRS	Katoonizadeh *et al*[177], Thabut *et al*[108]
Infection and sepsis	Sargenti *et al*[212], Bruns *et al*[164], Linderoth *et al*[213]

No well-established prognostic indicators are available for predicting ACLF outcome. Ambiguity in the diagnostic criteria has limited researches into the identification of clear indicators of severity. According to diverse population under study and definition of the syndrome, different indicators have been proposed. ACLF: Acute-on-chronic liver failure; INR: International normalized ratio.

Many studies suggest that HE is associated with higher mortality, especially in those with grade Ⅲ-Ⅳ encephalopathy[83,90,151,156]. This association is highlighted by the incorporation of HE to modified scores [*i.e.*, integrated-MELD (iMELD) score] with the aim of improving its predictive value[157,158]. In a recent study from Shi *et al*[159] when compared ACLF precipitated by hepatic insults to those precipitated by extrahepatic ones, the latter group had significantly higher 90-d and 1-year mortality; however, both groups had comparably high short-term mortality. This study also, demonstrates that the iMELD score may be a better predictor for hepatic-ACLF short-term prognosis, whereas CLIF-C-ACLF might be more beneficial for extrahepatic-ACLF patients. This novel score incorporates age and HE into MELD score, both, strong predictors of prognosis in hepatic-ACLF patients. The iMELD score has better predictive value of 3-mo mortality than the original MELD, SOFA, CLIF-SOFA and CPs in HBV-ACLF patients[158].

Recently, Wu *et al*[160] established and validated a new score to predict mortality risk in patients with HBV-ACLF. This score named "ALPH-Q score", integrates electrocardiography parameters, age, liver cirrhosis, prothrombin time and HE greater performance than CPs, MELD, and Logistic regression model (LRM) for predicting short-term mortality of patients with HBV-ACLF.

Many other factors summarized at Table 3 had been described.

MANAGEMENT

General management

At present, there is no ACLF-specific treatment. Current treatment consists of supportive measures, and therefore it should rely on enhanced care or intensive care units where the management of patients with multiorgan failure is protocolised, and patients can be closely monitored[107,140,161]. The aim of the general management should be focused on early recognition of any condition or precipitating factor which can cause ACLF, or, even more importantly, on avoiding exposure to those factors known to trigger multiple OFs. Although not proven, it is thought that the greatest impact on patient's outcome will be achieved by preventing or slowing a further progression of ACLF. Patients with ACLF present some unique features that may differentiate them from the non-cirrhotic patients and thus, a multidisciplinary approach is essential.

The main principle of treatment should therefore be to support organ function and treat precipitating factors while the liver recovers[6,12,25,45,162]. Treatment should be directed at addressing each specific dysfunction. For example, plasma expansion with albumin or crystalloids to improve the circulatory system or kidney function; administration of prednisolone in the setting of acute alcoholic hepatitis; renal replacement therapy to treat fluid, electrolyte, and acid-base abnormalities; vasoactive amines to improve ventricular function when circulatory failure or sepsis occurs; endotracheal intubation for airway control in patients with severe encephalopathy, in the presence of active upper gastrointestinal bleeding and/or lung failure. It should be noted, the great importance of both, prophylaxis and treatment of infections, given their crucial role of in the development of ACLF[66,69,144,163,164].

Liver support devices and liver transplantation

Liver support devices: When medical treatment fails, artificial liver support can be considered as a bridge therapy to liver transplantation or while the precipitating event is reversed. Yet organ shortage, cost, complications and side effects associated with immunosuppression, strongly limit this option. Furthermore, unstable clinical conditions of patients with ACLF are often contraindications for liver transplant.

Two types of devices can be distinguished; acellular devices such as albumin dialysis and plasma exchange [mainly molecular adsorbents recirculating system (MARS), and Prometheus devices], and cell-based devices, which incorporate cells from human, animal sources, or immortalized cells. The use of liver-assisting devices is based on their ability to remove toxic substances, inflammatory molecules, reduce NO, improve systemic hemodynamics and severe HE[165,166]. However, two prospective randomised studies, the RELIEF Study Group and the HELIOS

Study Group compared treatment with conventional therapy to MARS or to Prometheus, respectively, failed to show any survival benefit, despite improvement on biochemical parameters[167,168]. In contrast, Xu *et al*[169] and Ling *et al*[170] found that downgrading MELD in ACLF using these systems therapies improved the outcomes after liver transplantation.

Therefore, although these systems have some beneficial effects in patients with ACLF, their overall usefulness in this setting is uncertain. Until today, given the lack of acquiring a strict definition of ACLF, has undoubtedly made prospective studies in this field more difficult. Consensus on definition is needed to perform clinical trials able to translate liver assist devices application to a survival benefit in patients with ACLF[168,171-173].

Liver transplantation: Available information on liver transplantation for ACLF patients' is scarce, even though this represents the only definitive therapeutic option for the vast majority of patients with ACLF[174,175]. Nonetheless, as mentioned above, numerous reasons, including advanced age, active alcoholism, uncontrolled infections, concomitant diseases, and the presence of associated OFs, make patients with ACLF often unsuitable to undergo transplantation.

ACLF is associated with high short-term mortality rates of 50% to 90% and may evolve rapidly into a fatal clinical situation, thus the timeframe for evaluating patients and assessing them for LT is short[12,176,177]. More than 50% of the listed ACLF patients died on the waiting list which further demonstrates that the time period to transplantation is crucial, and that the window of opportunity is small[178]. Where the time of transplantation is a critical element in the patient's prognosis, living donor transplantation is an attractive alternative, since there are no waiting list constraints, and long-term survival has been shown to be comparable to living donor transplants[179-182]. There are limited evidences regarding the long-term outcome of patients transplanted for ACLF. Some studies showed similar survival rates of patients with ACLF to patients with chronic liver disease who underwent transplantation for other indications[174,178,183]. When interpreting these data sets, differences between western and eastern transplant centers must be taken into consideration.

Further studies are still necessary to determine timing of liver transplantation, optimal selection, and whether ACLF patients should be prioritized on a high-urgency list.

Antiviral therapy in ACLF

Antiviral therapy deserves particular mention due to the relevance of reactivation of HBV among aetiologies of ACLF in the Asia-Pacific region, where hepatitis-B-related cirrhosis constitutes around 70% of the underlying chronic liver diseases[30,105]. Furthermore, a large number of HBV-ACLF cases do not have underlying cirrhosis, as evidenced APASL ACLF Research Consortium (AARC) data based on the liver biopsy studies[184].

The aim of antiviral treatment for HBV-ACLF is to reduce viral DNA, so that reduction in hepatocyte cell death, helps prevent decompensation related multiorgan complications, and thereby improves survival outcomes[185-188]. Early treatment with nucleos(t)ide analogues such as lamivudine, tenofovir, entecavir or telbuvidine should be started[188-190]. Low pretreatment HBV DNA load and a rapid decrement in viral load improves outcomes in ACLF[191-194]. Some studies suggest that initial combination antiviral therapy is more effective than monotherapy[195-197].

New therapeutic targets

A few recent studies have tested the possibility of liver regeneration in a small group of patients with ACLF using granulocyte-colony stimulating factor therapy[192,198-201]. This cytokine mobilises bone marrow-derived stem cells and then restores neutrophil function, promotes hepatic regeneration, and thereby reducing the risk of developing kidney, or brain failure, and sepsis and thus improving survival of patients with ACLF. More studies are needed to provide clearer evidence.

Other proposed therapy for patients with ACLF, has been cell transplantation, either using hepatocytes or stem cells, to improve liver function thought cell repopulation of the liver and their potential anti-inflammatory effects, but again, these results await confirmation[202-205].

CONCLUSION

ACLF is a devastating syndrome since it remains a highly prevalent, life-threatening disease, which is clinically, pathophysiologically and prognostically a distinct entity from a mere decompensation of cirrhosis. In ACLF, altered host response to precipitating injury plays a pivotal pathophysiological role, such as SIRS. The degree of background immune paralysis and severity of OF determine the outcome of this syndrome. Ambiguity and variability among researcher groups on definitions criteria hampers precise characterization of this entity. Considerable efforts have been made to delve into the knowledge of this syndrome. Despite the progress, especially in pathophysiology, several questions remain: Fist, treatment strategies are currently limited to organ support; thereby a better understanding of underlying mechanisms will allow the development of new drugs and devices. Second, the absence of consensus on diagnostic criteria hampers the recognition of biomarkers and factors determining the outcome. Third, the most ambitious goal is, probably, the early recognition of this syndrome, in order to implement strategies to avoid the development of OF owing to

the reversibility of this profile of liver failure. Finally, a universally accepted definition is urgently needed.

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REVIEW

Is liver biopsy still needed in children with chronic viral hepatitis?

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Abstract

Liver biopsy is a standard method used for obtaining liver tissue for histopathological evaluation. Since reliable serological and virological tests are currently available, liver biopsy is no longer needed for the etiological diagnosis of chronic hepatitis B and C. However, liver histology remains the gold standard as a prognostic tool, providing information about the liver disease progression (grading of necroinflammatory activity and staging of fibrosis) and serving clinicians in the management and therapeutic decisions. In general, histopathological evaluation is indicated before starting the antiviral treatment. Main limitations of the liver biopsy include its invasive and painful procedure, sampling errors and the inter- and intra-observer variability. In addition, indications for the liver biopsy in pediatric patients with chronic viral hepatitis were questioned recently, and efforts have been made toward the development of non-invasive methods as an alternative to the liver biopsy. The most commonly used methods are novel imaging studies (elastography) and combinations of biomarkers. However, to date, none of these tests was validated in children with chronic viral hepatitis. In this review, we present the current status of the liver biopsy in the management of chronic viral hepatitis B and C in pediatric population, including specific indications, complications, contraindications, problems, limitations, and alternative non-invasive methods.

Key words: Liver biopsy; Hepatitis B; Hepatitis C; Pathology; Elastography; Fibrosis; Children

Core tip: The role of liver biopsy in pediatric patients with chronic viral hepatitis was questioned recently due

to the development of non-invasive alternative methods (novel imaging studies and combinations of biomarkers) used for the assessment of the severity of liver fibrosis. However, none of these methods has been validated in children so far, and therefore liver biopsy remains the gold standard for the evaluation of liver disease progression in children with chronic viral hepatitis. In addition, it is a crucial tool for the management and for therapeutic decisions.

Pokorska-Śpiewak M, Kowalik-Mikołajewska B, Aniszewska M, Pluta M, Marczyńska M. Is liver biopsy still needed in children with chronic viral hepatitis? *World J Gastroenterol* 2015; 21(42): 12141-12149 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i42/12141.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i42.12141

INTRODUCTION

Liver biopsy is a standard procedure used to obtain the liver tissue for histopathological evaluation[1,2]. Most commonly, it is performed percutaneously, without contemporaneous ultrasonographic guidance in determining the puncture site ("blind" liver biopsy, with a percussion-guided transthoracic approach, which is considered as the classic percutaneous method) or as ultrasound/computerized tomography-guided liver biopsy[1-3]. However, the role of ultrasonography in biopsy site determination is controversial. Among adult patients, ultrasonographic guidance was shown to be associated with decreased rates of hospitalization, but it did not influence rates of bleeding and hypotension[4,5]. Other less frequently used techniques include transjugular, plugged, and intraoperative or laparoscopic liver biopsy. Two main types of devices, available in different diameters, are used to obtain the liver tissue: suction and cutting needles. Liver biopsy is an invasive procedure. Thus, it is performed under general anesthesia or sedation in order to reduce pain and anxiety in patients[2]. Because of pediatric patients' lack of cooperation, general anesthesia is usually required[3]. Before performing the procedure, a written informed consent for the biopsy should be obtained from the patient and/or parents/guardians.

Three main indications for the liver biopsy include diagnostic and prognostic purposes, evaluating disease severity and monitoring response to treatment[2]. Liver diseases of different etiologies (*e.g.,* viral, autoimmune, nonalcoholic, and drug-induced hepatitis), as well as inherited metabolic diseases, cholestasis, liver tumors, acute liver failure, abnormal liver tests of unknown etiology, and others are considered as indications for liver biopsy in children. In recent years, due to the development of alternative methods of diagnosis of the liver diseases and advancement of imaging techniques (elastography), the role of the liver biopsy in chronic viral hepatitis has significantly evolved and is being questioned[2,3]. Thus, the aim of this review was to analyze the current status of the liver biopsy in the management of chronic viral hepatitis B and C in pediatric population, including specific indications, complications, contraindications, problems, limitations, and alternative non-invasive methods.

HISTOPATHOLOGICAL EVALUATION

In general, histopathological expression of the chronic viral hepatitis comprises the following three components: inflammation, fibrosis/cirrhosis, and hepatocellular changes[6]. Lesions typical for viral hepatitis, which enable the differential diagnosis with other chronic liver disorders, include portal tract inflammation consisting of mononuclear cells, common presence of interface hepatitis, usually focal lobular necrosis of variable degree, and mild bile duct damage (common in hepatitis C)[7,8]. Histopathological evaluation of the liver tissue in case of chronic viral hepatitis B or C should provide the following information: the extent of necroinflammation and fibrosis, the presence of any adjunctive lesions (steatosis, hemosiderosis, liver cell dysplasia), and detection of any comorbid conditions. In about 20% of patients with chronic hepatitis B or C, liver biopsy reveals other liver diseases which may affect disease progression and management (*e.g.,* non-alcoholic fatty liver disease)[7,9].

The extent of necroinflammatory activity and fibrosis has important implications for prognosis and therapy[7]. Fibrosis is considered as a better predictor of disease progression than necroinflammation[10,11]. The assessment of necroinflammatory activity and fibrosis is performed using several scoring systems, which take into account *grading* of the necroinflammatory activity and *staging* of fibrosis[7,12,13]. In 1981, Knodell *et al*[14] proposed the first semiquantitative scoring system - The Histological Activity Index (HAI). As HAI combined the necroinflammation and fibrosis, it is now rarely used in its original form and has been replaced by its modifications and other systems: Ishak, Scheuer, METAVIR, and Batts-Ludwig classifications[12,15-19]. All these systems are widely used in routine practice and for clinical trials, and there is no consensus as to which one is the best[7,20]. Clinicians should be familiar with the system used by the pathologist they cooperate with[7,20].

HEPATITIS B

Despite the implementation of universal immunization programs and blood-donor screening, infection with hepatitis B virus (HBV) is still one of the most important causes of liver disease. There are more than 360 million patients (6% of the population) suffering from chronic hepatitis B (CHB) worldwide with a significant number of children still being infected each year[21-24]. The clinical spectrum of CHB

Table 1 Indications for the liver biopsy in children with chronic hepatitis B according to the phase of the infection[21,22,29]

Phase of HBV infection	Immune-tolerant	Immune-active (-clearance)	Immune-inactive	Reactivation/HBeAg-negative chronic hepatitis
HBsAg	Detectable	Detectable	Detectable	Detectable
HBeAg	Detectable	Detectable	Undetectable (anti-HBe positive)	Undetectable (anti-HBe positive)
HBV DNA (IU/mL)/(copies/mL)	> 20000/> 10^5	> 20000/> 10^5	< 2000/< 10^4 or undetectable	> 2000/> 10^4
ALT	Normal	Persistently elevated	Normal	Normal or elevated
Histopathology: necroinflammation and fibrosis	Minimal or absent	Can develop	Liver inflammation absent or minimal, fibrosis regresses over time	Active liver inflammation +/- fibrosis
Liver biopsy	Generally not indicated	Indicated	Generally not indicated	Indicated, especially if ALT elevated
Antiviral therapy	Generally ineffective, risk of drug resistance; continued monitoring recommended	Should be considered	Continued monitoring recommended	Should be considered if moderate or severe inflammation or fibrosis detected

ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen.

in children ranges from asymptomatic carriage with minimal liver disease, to progression to cirrhosis and decompensated liver disease[25]. Despite a rather benign course of CHB during childhood, the lifetime risk of developing hepatocellular carcinoma (HCC) is 9%-24%, and the annual incidence of cirrhosis is estimated at 2%-3%[21,26,27]. Chronic HBV infection in childhood usually manifests as a mild liver disease; however, it can lead to cirrhosis in few, but not yet well identified cases[21,25,26,28,29]. The natural history of the disease is complex and, in general, consist of four phases: immune-tolerant, immune-active (-clearance), immune-inactive, and HBeAg-negative chronic hepatitis or reactivation[13,22,29] (Table 1). In children with CHB, liver histopathology evaluation remains crucial for the management of the liver disease, and hence the liver biopsy is essential before making treatment decisions and for predicting possible progression of the liver disease[30]. However, this procedure is performed only in a selected group of patients, based on the clinical evaluation[21,22,29]. Decision to start treatment in patients with CHB is based on alanine aminotransferase (ALT) level, HBeAg positivity, HBV DNA level, liver histology, family history of HCC, and other coexisting liver diseases[22]. In general, according to the current practical guidelines of the European Association for the Study of the Liver (EASL), and the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), liver biopsy is recommended in children with either persistently increased ALT levels and/or HBV DNA levels > 2000 IU/mL[22,23] (Table 1). In particular, before the initiation of the treatment, a histologic assessment of the necroinflammatory activity and the stage of fibrosis is recommended[22,29]. The response to the currently used antiviral drugs is more likely in patients with at least moderate necroinflammation or fibrosis[31,32]. But for children with mild histopathological features, such a benefit has not been established. However, if the child has a family history of HCC, the treatment should always be considered because of the increased risk for HCC development[22,33]. Liver biopsy is a useful tool in establishing prognosis and in predicting response to treatment, as more advanced necroinflammatory activity and fibrosis correlate with response to treatment using both interferon and nucleoside analogues[31,32]. Histological evaluation is also helpful in the diagnosis of cirrhosis, which is essential when interferon therapy is considered, as this may lead to decompensation of the liver disease in cirrhotic patients[3,34]. Liver biopsy findings in children with CHB are presented in Table 2.

HEPATITIS C

Hepatitis C virus (HCV) infection is considered as an important public health problem worldwide with an estimated global prevalence of 2.8% and 160 million people infected chronically[35,36]. Chronic hepatitis C (CHC) is a progressive disease, with 10%-20% of infected patients developing cirrhosis and about 7% of cirrhotic adult patients progressing to HCC[37,38]. In children and adolescents, CHC is usually described as a mild disease; however, severe cases with advanced fibrosis, cirrhosis, and even HCC in childhood have also been reported[39-43] (Table 2). It is estimated that about 5% of infected pediatric patients develop significant liver disease and 1.8% develop cirrhosis in childhood[39,44].

Since reliable serological and virological tests are currently available, liver biopsy is no longer needed for the diagnosis of CHC. The role of this procedure in the management of patients with CHC has also evolved with the development of non-invasive alternative methods and with the availability of the new, more effective treatment regimens, based on the direct-acting antivirals (DAAs). However, DAAs have not been implemented for routine practice in pediatric patients so far. Although histological inflammatory activity and fibrosis are likely to be mild in children with CHC, liver

Table 2 Liver biopsy findings in children with chronic hepatitis B and C *n* (%)

Type of infection	Patients (*n*)	Age,yr (mean ± SD or range)	Grading of necroinflammatory activity			Staging of fibrosis				Ref.
			mean HAI ± SD	Minimal/mild	Moderate/ severe	mean ± SD	No/low grade	Severe/ cirrhosis	Cirrhosis	
HBV	30	12.9 ± 2.5	5.4 ± 3.4	25 (84)	5 (16)	1.7 ± 0.9	24 (80)	6 (20)	1 (3)	Pokorska-Śpiewak *et al*[52]
HBV	35	10.2 (2.0-20.2)	-	33 (94)	2 (6)	-	28 (80)	7 (20)	2 (7)	Boxall *et al*[77]
HBV	190	7.5 ± 4.1	6.07 ± 3.22	135 (71)	55 (29)	1.71 ± 0.78	183 (96)	7 (4)	1 (0.5)	Mozer-Lisewska *et al*[30]
HBV	47	9 (1-17)	-	34 (72)	13 (28)	-	41 (87)	6 (13)	0	Dzierzanowska-Fangrat *et al*[78]
HCV	30	11.5 ± 3.6	4.2 ± 2.5	29 (97)	1 (3)	1.2 ± 0.9	28 (93)	2 (7)	0	Pokorska-Śpiewak *et al*[52]
HCV	44	8.6 ± 4.1	-	32 (73)	12 (27)	-	39 (89)	5 (11)	-	Mohan *et al*[43]
HCV	44	14.5 ± 4.0	-	33 (75)	11 (25)	-	35 (80)	9 (20)	-	Mohan *et al*[43]
HCV	112	8.6 (1-19)	-	-	-	-	107 (96)	5 (4)	1 (1)	Guido *et al*[79]
HCV	80	9.1 ± 4.8	-	62 (78)	17 (21)	-	66 (83)	13 (16)	1 (1)	Guido *et al*[80]
HCV	121	9.8 ± 3.7	5.1	72 (60)	49 (40)	-	114 (94)	7 (6)	2 (2)	Goodman *et al*[46]
HCV	42	13.4 ± 4.1	-	30 (71)	12 (29)	-	37 (88)	5 (12)	-	Mohan *et al*[42]
HCV	109	8.8 ± 4.2	3.3 ± 1.5	-	-	1.36 ± 0.5	105 (97)	4 (3)	-	Kage *et al*[81]
HBV/ HCV	10	12.6 ± 2.7	6.2 ± 3.0	7 (70)	3 (30)	1.7 ± 0.8	9 (90)	1 (10)	0	Pokorska-Śpiewak *et al*[52]

HAI: Histological activity index; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 3 Indications for the antiviral treatment in patients with chronic hepatitis C according to the stage of fibrosis[45]

Stage of fibrosis (METAVIR)	Treatment
Significant fibrosis (F3) or cirrhosis (F4), including decompensated cirrhosis	Should be prioritized
Moderate fibrosis (F2)	Is justified
No or mild liver disease (F0, F1)	Can be deferred

biopsy is recommended by EPGHAN as a baseline investigation before the HCV infection treatment in clinical trials[44]. According to the recent EASL Recommendations on Treatment of Hepatitis C (2015), before the initiation of the therapy, the assessment of liver disease severity should be performed[45]. As the post-treatment prognosis depends on the stage of fibrosis, identifying patients with advanced fibrosis or cirrhosis is particularly important. If significant fibrosis is absent, the timing of therapy is possible (Table 3). The evaluation of liver disease severity is important regardless of ALT levels, as significant fibrosis may also be present in patients with repeatedly normal ALT[45,46]. For many years, for the assessment of liver disease severity in patients with CHC, liver biopsy was the only method of choice. On the contrary, at present time, according to the recent EASL guidelines[45], in patients with CHC, the stage of fibrosis can be assessed prior to the treatment by non-invasive methods: liver stiffness measurement or well-established panels of biomarkers. However, these methods perform well in identifying cirrhosis or no fibrosis but are less reliable in resolving intermediate degrees of fibrosis. The combination of both methods (liver stiffness measurement and a blood test) may improve their accuracy and reduce the need for liver biopsy to resolve uncertainty[47,48]. Castera[49] proposed an algorithm for treatment-naïve patients with CHC, that combines two unrelated non-invasive methods: transient elastography (TE) and serum biomarker as first-line assay of fibrosis stage. According to this algorithm, liver biopsy might be necessary in patients infected with HCV genotype 1 or 4, before the treatment, if the results of TE and biomarker test are discordant[49]. Histopathological evaluation is also needed in cases of potential additional etiologies (HBV infection, metabolic syndrome, alcoholism or autoimmunity)[45]. It is essential to identify the adjunctive liver lesions like autoimmune hepatitis (especially in patients with positive LKM1 autoantibodies) or steatosis, which is a prognostic factor for the treatment response[44,50]. In pediatric patients, although several non-invasive tests alternative to liver biopsy have been investigated, none of them was validated so far and therefore this recommendation of EASL to replace liver biopsy by non-invasive tests should be approached with caution in children with CHC.

Antiviral treatment is indicated in all treatment-naive and treatment-experienced patients with compensated and decompensated liver disease due to HCV infection[45]. However, some prioritization of patients is necessary. One of the main factors considered is liver fibrosis (Table 3).

COINFECTIONS

Coinfection with HBV/HCV is usually associated with more severe liver disease, and with a frequent progression to cirrhosis and HCC compared to the monoinfection with either virus[13]. However,

there is some evidence on a reciprocal replicative suppression between both viruses[13,51]. A recent study on pediatric patients showed that HBV/HCV coinfection is an independent predictor of moderate-to-severe necroinflammatory activity[52] (Table 2). In patients with CHB, the HDV coinfection may lead to more severe liver disease with accelerated fibrosis progression, an earlier hepatic decompensation, and an increased risk for HCC[13,53]. A potential or confirmed mixed etiology of the liver disease is considered as an indication for a liver biopsy and histopathological evaluation[45,50].

COMPLICATIONS AND CONTRAINDICATIONS

In general, complications after liver biopsy are rare[3]. It is estimated that 0.9% patients suffer from complications requiring hospitalization associated with percutaneous liver biopsy[13]. The most commonly reported complication is transient and localized abdominal pain and/or right shoulder discomfort, which occur in 20%-84% of patients[3,54]. Pain after liver biopsy is usually mild, well tolerated, and easily controlled by minor analgesia[2]. Major complications occur with the prevalence of 0%-4.6% and include: hemorrhage, pneumothorax, hemothorax, visceral perforation, cholangitis, bile leak, bile peritonitis, hemobilia, infection, arteriovenous fistula, neuralgia, sedation-related injury[1,3]. The risk of death following liver biopsy in adults is estimated at 1:10000 cases[2]. In one study performed in children, 3 deaths have been reported among 469 patients (0.6%), all these patients had a history of malignancy or hematological disease[55]. In other two recent studies, no death was reported among pediatric patients[56,57].

Contraindications for liver biopsy are rather relative than absolute. The most common contraindication is severe coagulopathy. There are no specific cutoffs for laboratory parameters for impaired hemostasis, and every center at which liver biopsy is performed should define ranges for coagulation parameters that either preclude liver biopsy or require blood product administration[1,3]. However, INR > 1.5 and platelet count < 60000/mL usually indicate an increased risk of bleeding[1,3]. In case of high-risk patients (with coagulopathy and severe liver disease, pancytopaenia, or clinically evident ascites ascites) and in patients with contraindications to percutaneous liver biopsy (*e.g.*, haematological conditions), a transjugular instead of percutaneous approach is recommended[1,2]. Morbid obesity, possible vascular lesions, extrahepatic biliary obstruction, and bacterial cholangitis are other potential contraindications to percutaneous liver biopsy[1,3].

PROBLEMS AND LIMITATIONS

Since liver biopsy is an invasive procedure, it is frequently more complicated and more expensive in pediatric patients compared to adults. There are often technical problems with obtaining appropriate tissue specimen because of the size of the patient and/or liver[3]. In chronic hepatitis, sample size can affect the diagnostic accuracy of liver biopsy specimen because it is estimated that the biopsy represents approximately only 1/50000 of the total mass of the liver[10]. Thus, the sampling error can approach 20%-30%[3]. It is estimated that at least 11 complete portal tracts and a biopsy specimen of at least 20 mm length are required for an accurate diagnosis[58,59]. Bedossa *et al*[60] demonstrated that for reliable staging of fibrosis in patients with CHC, a 25 mm biopsy specimen length is adequate to overcome variation due to sampling. In pediatric patients it is frequently not achieved due to the size of the patient or liver.

Another important issue is the interpreter. There is a well-recognized possibility of inter- and intra-observer variability in the assessment of liver biopsy specimen, which may be a major potential limiting factor in liver biopsy interpretations[61,62]. Diagnostic errors made by pathologists without specialty experience in pediatric liver diseases were reported in more than 25% of samples[63]. However, when the pathologist interpreting the specimen has subspecialty expertise in liver histopathology and over 10 years of experience at an academic center, it leads to improved consistency and accuracy, minimizing problems related to the sample size[64].

ALTERNATIVES TO LIVER BIOPSY

Limitations of liver biopsy have paved the way for the development of new alternative non-invasive methods of evaluation of liver disease. In case of chronic viral hepatitis, novel imaging studies (elastography) and combinations of biomarkers are used.

Elastography enables determination of liver fibrosis by measuring liver tissue mechanical properties, in particular its stiffness (elasticity), which is reduced in case of fibrosis[1,3,65]. Elastography techniques include TE, acoustic radiation force impulse imaging (ARFI), shear wave elastography (SWE), and magnetic resonance elastography (elasto-MR). TE is the most commonly used method based on shear wave, which is generated by an external mechanical impulse. Its speed is measured by an ultrasound one-dimensional probe. The elasticity is measured at depth ranging from 25 mm to 65 mm in a 1 cm × 4 cm area, which makes the assessed liver volume two hundred times greater than that examined during the liver biopsy[65]. Since 2008, liver stiffness can be measured also in small children, since a new probe with a smaller diameter (S-probe 5 mm) compared to the regular probe (M-probe 7 mm) is available. ARFI is a new method for quantifying elasticity of tissue by measuring the shear wave velocity induced without manual compression, but using acoustic radiation

Table 4 Diagnostic performance of the main non-invasive methods used to determine significant liver fibrosis (METAVIR F ⩾ 2) and cirrhosis (METAVIR F4) in adult and pediatric patients

Test	Patients (*n*)	Disease	AUROC		Ref.
			F ⩾ 2	F4	
Fibrotest	3501	HCV	0.85	-	Poynard *et al*[82]
Fibrotest	1457	HBV	0.80	-	Poynard *et al*[82]
Fibrotest	116[1]	Chronic liver disease	-	0.73	de Lédinghen *et al*[69]
APRI	6259	HCV	0.77	0.83	Lin *et al*[83]
APRI	116[1]	Chronic liver disease	-	0.73	de Lédinghen *et al*[69]
TE	251	HCV	0.79	0.97	Ziol *et al*[84]
TE	183	HCV	0.83	0.95	Castéra *et al*[47]
TE	165	HCV	0.88	0.90	Nitta *et al*[85]
TE	400	HCV	0.818	0.932	Sporea *et al*[86]
TE	173	HBV	0.81	0.93	Marcellin *et al*[87]
TE	175	HBV	0.95	0.98	Zhu *et al*[88]
TE	116[1]	Chronic liver disease	-	0.88	de Lédinghen *et al*[69]
TE	30[1]	HCV	0.815	1.00	Awad *et al*[89]
ARFI	911	HCV	0.792	0.842	Sporea *et al*[86]

[1]Children. AUROC: Area under the receiver operator characteristic curve; APRI: Aspartate-to-platelet ratio index; ARFI: Acoustic Radiation Force Impulse Imaging; TE: Transient elastography; HCV: Hepatitis C virus.

propagating in the tissue. ARFI provides a single one-dimensional measurement of tissue elasticity and is performed using a conventional ultrasound diagnostic device. SWE is a novel method introduced in 2005, based on the generation of a radiation force and measuring the shear wave propagation speed in the liver tissue. SWE provides a real time two-dimensional map of tissue elasticity and, as ARFI, it is incorporated into a conventional ultrasound diagnostic device. Elasto-MR is a technique, which can diagnose severe fibrosis or cirrhosis with high accuracy[66]; however, it is an expensive and currently not available method for the clinical use[1,3].

Many combinations of serum biomarkers, measured in routine blood tests, were evaluated for their ability to indicate alterations in hepatic function and to determine stage of liver fibrosis[49]. The simplest one is aspartate-to-platelet ratio index (APRI). In case of chronic viral hepatitis, the most commonly used biomarker test is Fibrotest, which combines alpha-2 macroglobulin, haptoglobin, GGT, apolipoprotein A1, and total bilirubin serum levels[67].

The diagnostic performance of non-invasive methods is evaluated by calculation of the area under the receiver operator characteristic curve (AUROC), with liver biopsy as a reference standard. An analyzed method is defined as being perfect when the AUROC is 100%, excellent if AUROC is over 90%, and good if AUROC is over 80%[49,65]. In clinical studies, detection of significant fibrosis (METAVIR F ⩾ 2) and detection of cirrhosis (METAVIR F4) are considered as relevant end points[49]. The non-invasive methods have been evaluated for their ability to determine stage of liver fibrosis mainly in adult patients with CHC and less frequently with CHB. Data regarding evaluation of this methods in children are only sparse and inconsistent (Table 4). In adults, different elastography methods show sensitivity and specificity of almost 90% in detecting advanced fibrosis[68]. In limited pediatric studies, TE accurately discriminated patients with severe fibrosis or cirrhosis from those without fibrosis[69,70]. However, elastography does not enable differentiation between stages of fibrosis and, to date, only a few studies have correlated liver stiffness as assessed by elastography with histological staging of fibrosis in pediatric patients[71]. In addition, the role of this method is limited in patients with edema, inflammation, extrahepatic cholestasis, and congestion, which can also dampen elasticity[72]. The role of biomarker tests was analyzed in several cohorts of patients, including children[69,73-76]. As for the liver stiffness measurement, biomarkers identify the cirrhosis or no fibrosis, but they fail to resolve intermediate degrees of fibrosis[45]. In addition, there is some evidence on discordance between Fibrotest and METAVIR scores in children with CHC and CHB[75,76]. According to the international experts, the non-invasive methods used to assess the stage of liver fibrosis are still not fully validated; they do not evaluate necroinflammatory activity, and therefore cannot substitute for liver biopsy in children with chronic viral hepatitis[22].

CONCLUSION

For the evaluation of necroinflammation and fibrosis in pediatric patients with chronic viral hepatitis, liver biopsy remains a gold standard despite its invasive procedure. Until the non-invasive methods of grading and staging of the chronic liver disease in children are fully validated, histological evaluation remains crucial for monitoring liver disease severity and for therapeutic management decisions. Further prospective studies on larger cohorts of pediatric patients are required

before liver biopsy could be replaced by non-invasive methods in children suffering from chronic HBV or HCV infection.

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REVIEW

Oncogenic role of p21 in hepatocarcinogenesis suggests a new treatment strategy

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Abstract

A well-known tumor suppressor, p21, acts paradoxically by promoting tumor growth in some cellular conditions. These conflicting functions have been demonstrated in association with the *HBx* gene and in hepatocarcinogenesis. The molecular behavior of p21 depends on its subcellular localization. Nuclear p21 may inhibit cell proliferation and be proapoptotic, while cytoplasmic p21 may have oncogenic and anti-apoptotic functions. Because most typical tumor suppressive proteins also have different effects according to subcellular localization, elucidating the regulatory mechanisms underlying nucleo-cytoplasmic transport of these proteins would be significant and may lead to a new strategy for anti-hepatocellular carcinoma (HCC) therapy. Chromosome region maintenance 1 (CRM1) is a major nuclear export receptor involved in transport of tumor suppressors from nucleus to cytoplasm. Expression of CRM1 is enhanced in a variety of malignancies and *in vitro* studies have shown the efficacy of specific inhibition of CRM1 against cancer cell lines. Interestingly, interferon may keep p21 in the nucleus; this is one of the mechanisms of its anti-hepatocarcinogenic function. Here we review the oncogenic property of p21, which depends on its subcellular localization, and discuss the rationale underlying a new strategy for HCC treatment and prevention.

Key words: p21; Tumor suppressors; Oncogene; Subcellular localization; Hepatocellular carcinoma; HBx; Nucleo-cytoplasmic export; Chromosome region maintenance 1; Selective inhibitors of nuclear export; Interferon

Core tip: A well-known tumor suppressor, p21, can act paradoxically by promoting tumor growth, depending on its subcellular localization. Nuclear p21 may inhibit cell proliferation while cytoplasmic p21 may be associated with anti-apoptotic and oncogenic functions.

These conflicting roles are reviewed in the context of the *HBx* gene and hepatocarcinogenesis. Because most tumor suppressors act in a similar manner to p21, regulation of their nucleo-cytoplasmic export, which is mainly effected *via* chromosome region maintenance 1, may be a basis for developing a new strategy for anti-hepatocellular carcinoma therapy.

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INTRODUCTION

Primary liver cancer is the 5th most common cancer in men and the 7th in women, with high mortality worldwide; therapeutic options for cure are urgently needed[1,2]. Hepatocellular carcinoma (HCC) accounts for most primary liver cancer. Although there is a geographic difference in incidence of HCC caused by some etiological variation, the major etiological agents are hepatitis B (HBV) and hepatitis C virus (HCV) infection. Once these viruses infect liver, they ingeniously evade host immune surveillance and induce chronic necroinflammation, leading to fibrosis and, ultimately, liver cirrhosis. Hepatocytes, *via* their innate regenerative capacity, continue to proliferate in order to compensate for the necrotic tissue. Genetic alterations continuously accumulate during these processes, resulting in pathogenic liver changes such as cirrhosis from which HCC frequently arises[3]. Once HBV-related cirrhosis is established, HCC develops at an annual rate of about 4% in Japan, for example[4].

Several lines of evidence support the direct involvement of HBV in the transformation processes. HBV is like a retrovirus in that it integrates into the host genome, causing chromosomal abnormalities. In addition, the *HBx* gene acts like an oncogene by trans-activating many genes involved in cellular transformation.

No common molecular mechanisms that account for the extremely complex process of hepatocarcinogenesis have yet been elucidated. Genetic alterations reported have been heterogenous, involving abnormalities of many signal transduction pathways[2,3]. However, a fundamental abnormality in hepatocarcinogenesis, like other malignancies, is deregulation of the cell cycle. The main regulators of the cell cycle are cyclin-dependent kinase inhibitors (CDKI), such as p21, p27, and p16, widely known as tumor suppressors. However, it is noteworthy that these tumor suppressors can function in an oncogenic manner depending on their precise intracellular localization. In this review, we explore the relevance of the intracellular localization of p21, in particular, and its function, to highlight the possibility that regulating the intracellular localization of tumor suppressors may be a potential future anti-HCC strategy in the context of both directly-killing tumor cells and preventive role.

P21 AS AN ONCOGENE

First identified in 1993[5,6], p21 is a universal CDKI that causes G1 growth arrest downstream of p53[7,8]. p21 binds to CDKs and inhibits the kinase activity, leading to growth arrest at specific stages in the cell cycle[9,10]. p21 also induces cellular differentiation and senescence.

Although p21 is one of the major tumor suppressors, it also can promotes oncogenesis. High expression of p21 is associated with poor prognosis of cancer[11-13]. Although mutation of the *p21* gene has been reported in bladder cancer[14], most reported studies failed to show the loss-of-function mutations of p21[15-17]. These results suggest that p21 may not be a classical tumor suppressor.

Experimental results of using genetically-engineered mice also support conflicting functions of p21. Spontaneous tumors occurred in p21-deficient mice, providing evidence that p21 is a tumor suppressor[18]. p21 also causes genomic instability[19]. However, the timing of tumor formation in p21-deficient mice was later than p53-deficient mice[20]. Moreover, the occurrence of lymphoma was suppressed when p21-deficient mice were crossed with p53- or ATM-deficient mice[21,22]. This result indicates that p21 also acts in an oncogenic way in particular conditions, reflecting its versatile function[9]. In addition, mammary gland tumorigenesis was accelerated in mice in which p21 was overexpressed in cytoplasm[23], and the cyclin-binding motif of p21 has been reported to have a direct tumorigenic function[24].

HCC, HBX AND P21

There have been many reports regarding the expression of p21 in HCC tissues. p21 was found to be down-regulated in HCC tissues, demonstrating its tumor suppressive function[25-27]. Kao *et al*[28] also reported that p21 expression was observed in 37% of HCC tissues, regardless of p53 expression, and was an independent survival good prognosis factor. While most of the reports show that p21 acts as a tumor suppressor, expression levels of p21 in liver cirrhosis have been reported to be correlated with the cumulative incidence of the occurrence HCC[29] and to be dominant in cytoplasm when histology became more undifferentiated[30].

There are also some reported studies examining the relationship between HBV and p21. Some reports have shown that the *HBx* gene exerts oncogenic activity by suppressing p21 expression[31,32] and that HBx genes having core promoter mutations suppress p21 more effectively[33]. Inversely, HBx enhanced p21

in some reports[34,35]. Park *et al*[34] reported that when the cell cycle was prolonged by enhancement of p21 by HBx, cells had survival advantages and chances for gene mutations, eventually leading to preneoplastic hepatocytes. In addition, Yano *et al*[36] reported that HBx enhanced cytoplasmic p21 in protein kinase C (PKC)-dependent manner to induce cell proliferation. These conflicting results may partly come from differences in experimental conditions, but mostly reflect the conflictive function of p21.

ASSOCIATION BETWEEN MOLECULAR BEHAVIOR OF P21 AND ITS ONCOGENIC FUNCTION

What molecular behavior of p21 does correlate with its tumor-promoting function? It is well-known that p21 has not only inhibitory effects on cell cycle, but also has a promoting role. p21-associated CDKs exist in both active and inactive states[37]; p21 promotes the assembly of CDK4,6 and cyclin D and exerts oncogenic activity without inhibiting kinase activity[38]. Mantel *et al*[39] found a high level of induction of p21 in a myeloid cell line that was induced to proliferate by growth factors. p21 also induces nuclear retention of cyclin D1, inhibiting its cytoplasmic degradation[40]. p21 induces cell cycle progression in glioma[41] and in vascular smooth muscle cells[42] by promoting the formation of active cyclin-CDK complexes with PKC-alpha. Because the lymphoma observed in p21-deficient mice has high levels of apoptosis[21], the oncogenic activity of p21 may be closely associated with its anti-apoptotic function

P21 has a dual function with regard to apoptosis. p21 halts the cell cycle and prevents apoptosis induced by genotoxic agents. This anti-apoptotic function of p21 may be associated with its oncogenic property. However, p21 acts as a pro-apoptotic in some conditions. Forced expression of p21 induces the apoptotic response against cisplatin in glioma[43] and in ovarian cancer[44]. p21 is a modulator of apoptosis in a p53-dependent or -independent manner[10]. Masgras *et al*[45] reported that cell-specific sensitivity to oxidative stress determined whether the cell was fated to undergo p21-induced cell death.

ACTIONS OF P21 DEPEND ON SUBCELLULAR LOCALIZATION

The dual functions of p21, apoptotic or anti-apoptotic, depend on its subcellular localization[9,46]. Nuclear p21 is anti-proliferative and cytoplasmic p21, which is anti-apoptotic, may be associated with oncogenic function. Cytoplasmic p21 is associated with poor prognosis or the aggressiveness of human cancer[11,12,30,47]. Cytoplasmic localization of p21 is closely associated with the phosphorylation status. Phosphorylation at Thr57 and Ser130 by extracellular signal-regulated kinase (ERK) inhibits nuclear localization of p21 and causes its cytoplasmic accumulation, inducing cell cycle progression[48]. Phosphorylated p21 that locates in cytoplasm has anti-apoptotic action by inhibiting the apoptotic proteins. Koster *et al*[49] reported that cytoplasmic p21 conferred resistance against cisplatin-induced apoptosis, while it became pro-apoptotic when it entered in the nucleus by the inhibition of AKT. Involvement in signal transduction of phosphorylated p21 differs depending on the site of the phosphorylated amino acid; Thr145 by AKT[50,51] or Ser130 by p38 and JNK[52]. Cytoplasmic p21 prevents apoptosis by inhibiting procaspase 3[53], and apoptosis signal-regulating kinases (ASK) 1[54].

As a summary, p21 as a tumor suppressor may be associated with nuclear location that may be associated with inhibition of cell proliferation and pro-apoptotic function, while oncogenic, anti-apoptotic p21 may require a cytoplasmic location. Thus, the shifting subcellular localization of p21 may be the clearest way to explain its functional versatility.

It is well known that not only p21 but most tumor suppressive proteins have different effects in different subcellular compartment. Cancer cells respond to what are typically tumor suppressors, such as p21, Rb, p53, p27, breast cancer susceptibility gene (*BRCA*) 1 and *FOXO* (forkhead box-containing, O subfamily) by proliferating when these molecules relocate from nucleus to cytoplasm[55,56]. Thus, based on the discussion above regarding p21, it is possible to extend this view to tumor suppressors in general. Regulating the subcellular localization of these proteins may become a core rationale for anti-cancer strategy[55,56].

REGULATION OF THE SUBCELLULAR LOCALIZATION OF TUMOR SUPPRESSORS AND ITS POTENTIAL APPLICATION TO ANTI-HCC TREATMENT

Transport of macromolecules across the nuclear envelope occurs through nuclear pore complexes (NPC). Karyopherins, such as exportins and importins, are nuclear transport receptors that recognize nuclear export signal (NES) and nuclear localization signal (NLS) sequences, respectively, and transport cargo proteins at the NPC sites[55,57]. Subcellular localization of tumor suppressors is regulated by this nucleo-cytoplasmic transport system[55,56,58,59].

CRM1 (Exportin-1/chromosome region maintenance 1) is a major nuclear export receptor that forms NPC with nucleoporins, such as NUP214 and NUP88, transporting nuclear proteins with NES sequence to cytoplasm[55-59]. CRM1 is deeply involved in the mechanisms of cell proliferation by regulating the subcellular localization of tumor suppressors which have NES sequences, such as p53 and p21. For example, p53

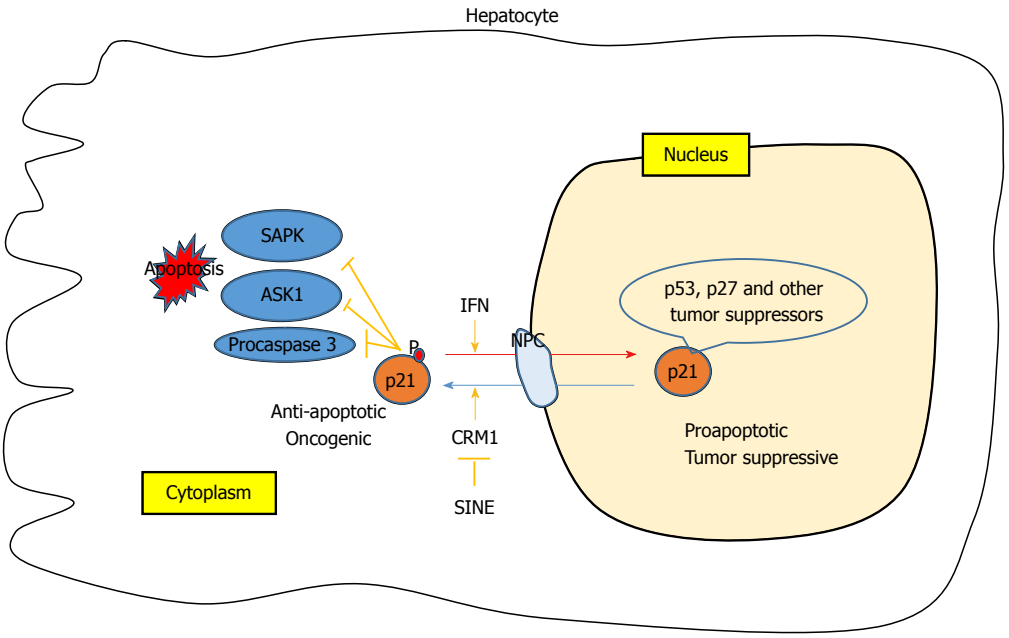

Figure 1 Outline of the overall aspects of this review. The subcellular localization of p21 and other tumor suppressors is regulated by CRM1. Inhibition of CRM1 by specific inhibitors and IFN may play a role in future anti-hepatocellular carcinoma strategies. CRM1: Chromosome region maintenance 1; IFN: Interferon; SINE: Selective inhibitors of nuclear export.

accumulates in the nucleus when poly (ADP-ribosyl)ation blocks its interaction with CRM1[60], while interaction with SUMO (small ubiquitin-like modifier) promotes its export to cytoplasm by CRM1[61]. p21 inhibits CRM1 by binding phosphorylated cyclin D, which promotes its nuclear accumulation[40].

Cancer cells use nucleo-cytoplasmic transport system for their proliferation and inhibition of apoptosis. CRM1 expression is enhanced in many cancer tissues. High expression of CRM1 in gastric, ovarian, and pancreatic cancers show poor prognosis[62-64]. The increase in CRM1 leads to cytoplasmic abundance of tumor suppressors and cell cycle regulators, which in turn results in their aberrant activation. Knock-down of CRM1 expression prevents nuclear export of p27, resulting in cell cycle arrest[65]. Specific suppression of CRM1 caused nuclear retention of p21 and induced apoptosis[66]. The cellular apoptosis susceptibility (CAS)/importin pathway was found to be enhanced in HCC, confirming the importance of the transport machinery[67].

Orally available selective inhibitors of nuclear export (SINEs) which specifically inhibit CRM1, have been developed in recent years[58,59]. SINEs specifically bind Cys528, located in NES-binding groove of CRM1, to promote nuclear retention of p53, p21, p27, Rb, and BRCA 1[68]. The effects on hematologic malignancies of KPT-330, the most effective SINE, have been reported[69-73]. KPT-330 had anti-proliferative effects and induced apoptosis of an HCC cell line[74] in which p53-upregulated-modulator of apoptosis was markedly up-regulated; and this was shown to be one of the similar mechanisms by which sorafenib exerts anti-HCC effects[75].

Interestingly, interferon (IFN)-beta was reported to return cytoplasmic p21 to the nucleus and contributed to the prevention of hepatocarcinogenesis[36]. This was also true of p53 that was bound to cytoplasmic HBx and returned to the nucleus after IFN-treatment[76] (Figure 1). These observations suggest that natural substances such as IFN may be involved in an innate carcinogenesis prevention mechanisms, possibly by regulating CRM1. In fact, CRM1 is involved in the cytoplasmic localization of STAT2, which shifts to the nucleus by the action of IFN[77]. In addition, IFN inhibits beta-catenin signaling through the up-regulation of the nuclear RanBP3 which is a nuclear export factor[78].

FUTURE PERSPECTIVE

Sorafenib is a tyrosine kinase inhibitor widely used for the treatment of advanced HCC, and many other molecular-targeted drugs are now in development[79]. However its effect is still limited in many patients and the appearance of drug-resistance is a significant problem. Regulation of CRM1 involves many genes and specific multiple pathways associated with nuclear-cytoplasmic export; a new therapeutic strategy could be based on these developing concepts. Because such regulation would normalize molecular changes caused by multiple genes, its use might not cause drug resistance, or even being suggested to reverse drug resistance[55]. Thus, combination of such regulation with specific inhibitor use might maximize the impact of treatment. While expecting some promising results of clinical trials, taking the molecular approach to explore innate mechanisms regulating nuclear-cytoplasmic distribution of tumor suppressors will become an intriguing theme for development of cancer prevention strategies. In particular, IFN might

influence these mechanisms and play a role in anti-hepatocarcinogenesis. Future uses of this drug should be pursued in light of this functional biological aspect.

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REVIEW

Transposon mouse models to elucidate the genetic mechanisms of hepatitis B viral induced hepatocellular carcinoma

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Abstract

The major type of human liver cancer is hepatocellular carcinoma (HCC), and there are currently many risk factors that contribute to this deadly disease. The majority of HCC occurrences are associated with chronic hepatitis viral infection, and hepatitis B viral (HBV) infection is currently a major health problem in Eastern Asia. Elucidating the genetic mechanisms associated with HBV-induced HCC has been difficult due to the heterogeneity and genetic complexity associated with this disease. A repertoire of animal models has been broadly used to study the pathophysiology and to develop potential treatment regimens for HBV-associated HCC. The use of these animal models has provided valuable genetic information and has been an important contributor to uncovering the factors involved in liver malignant transformation, invasion and metastasis. Recently, transposon-based mouse models are becoming more widely used in liver cancer research to interrogate the genome by forward genetics and also used to validate genes rapidly in a reverse genetic manner. Importantly, these transposon-based rapid reverse genetic mouse models could become crucial in testing potential therapeutic agents before proceeding to clinical trials in human. Therefore, this review will cover the use of transposon-based mouse models to

address the problems of liver cancer, especially HBV-associated HCC occurrences in Asia.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Transposable elements; Sleeping Beauty; Forward and reverse genetic screens

Core tip: Hepatocellular carcinoma (HCC) is the major type of primary liver cancer and the risk factors that contribute to its formation are hepatitis viral infection, alcohol consumption, aflatoxin exposure, hemochromatosis, and tyrosinemia. *In vivo* forward and reverse genetic transposon models have been used to study the genetic mechanisms of HCC, including hepatitis B viral-induced HCC. These animal models provide valuable genetic information and are important contributors to uncovering the factors involved in liver malignant transformation, invasion and metastasis. They could also be used to test potential therapeutic agents before proceeding to clinical trials in humans.

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EPIDEMIOLOGY OF LIVER CANCER

Liver cancer is the second most common cause of cancer related death worldwide, accounting for about 700000 deaths annually. Liver cancer has a poor prognosis, with less than 20% of advanced staged patients surviving for more than one year after initial detection[1]. The major type of primary liver cancer is hepatocellular carcinoma (HCC), which accounts for 85% to 90% of total liver cancer cases[2-4]. In 2012, 83% of 782000 new liver cancer cases worldwide occurred in Eastern Asian countries like Korea, China, Japan, as well as sub-Saharan, middle and Eastern Africa, with a high incidence rate of more than 20 per 100000 individuals. The incidence rate is particularly high in China, with 50% of the total new liver cancer cases diagnosed there[2-6]. Middle Africa, Central America, North America, and Southern Europe have a moderately high incidence rate of about 10 to 20 per 100000 individuals, while a low incidence rate has been reported in South-Central Asia and Northern Europe with less than 5 liver cancer cases per 100000 individuals[2-4,6].

HCC most often is diagnosed between the ages of 55 to 59 years old in China, and between 63 to 65 years old in Europe and North America[3,4]. HCC is a gender-biased disease more commonly found in males than in females with the ratio of about 3:1 in high incidence rate countries. According to long historic studies, hormonal differences between males and females are one of the reasons for this gender bias phenomenon. Researchers have suggested the androgen receptor (AR), which can bind to testosterone and other male hormones and is more highly expressed in males than in females, can promote hepatitis B virus (HBV)-related HCC, while estrogen receptor 1 (ESR1) may work antagonistically[7-10]. The activation of *Esr1* has been shown to dampen hepatitis B viral replication[7], and estrogen can also protect female mice by repressing the expression of interleukin 6 (*Il6*) in Kupffer cells and resident macrophages in liver to prevent liver inflammation[7,8].

HBV INFECTION

HBV infection is a major global health problem and, according to the World Health Organization, more than 300 million people suffer from chronic HBV infection and about 780000 people die every year due to acute or chronic consequences of the disease[4,11]. Since 1970, chronic HBV infection has been shown to be closely related to the development of liver cirrhosis and HCC[12]. The median survival rate for HBV-associated HCC is less than 16 mo, and the five year survival rate is only 15% to 26%[4].

In developing countries, chronic HBV infection accounts for most HCC cases with HBV mostly being transmitted from mother to infant and approximately 90% of infants born to chronic HBV-infected mothers eventually developing the disease[4]. In contrast, HCV infection-associated cirrhosis and alcoholic cirrhosis account for the majority of HCC cases in developed countries that have access to HBV vaccination programs[4]. In countries with these vaccination programs, HBV is usually transmitted sexually between adults and 90% of the virus can be spontaneously cleared by the infected host's immune system[4]. Although vaccines for HBV have been introduced to reduce the prevalence of HCC, HCC occurrence has not declined in the last two decades due to high chronic HBV infection prevalence and prolonged HCC development latency[4].

HBV GENOTYPES

As of 2011, 10 HBV genotypes (A to J) have been identified and classified by their infectious geographical distribution. Genotype A is widely distributed in South and West Africa and in Western and Northern Europe. Genotypes B and C are commonly found in East and Southeast Asia, including China, while genotype D is distributed mainly in the Mediterranean area[4]. Any genomic variations of HBV, such as genotypes, sub-

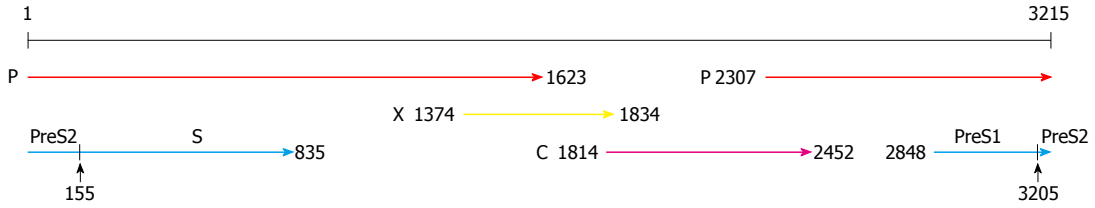

Figure 1 Structure of the hepatitis B virus genome. The hepatitis B virus genome consists of an incomplete double stranded DNA with four open reading frames: surface proteins encoding gene (PreS1/S2/S), polymerase encoding gene (P), X protein encoding gene (X), and core protein encoding gene (C)[17].

genotypes, and HBV gene mutations can further contribute to the development of liver cirrhosis and HCC in patients with different HBV replication and hepatitis B surface antigen (HBsAg) status[4,5]. Prolonged expression of HBV correlates with the development of HCC, likely due to the presence of its viral proteins such as the X gene (HBx) and/or large envelope proteins. These viral proteins can activate cellular cancer-related genes, induce oxidative stress, and promote genetic instability that further contributes to hepatocyte transformation[13]. HCC tumorigenesis is also influenced by the patient's viral status such as the HBV genotype, hepatitis B e antigen (HBeAg) serostatus and mutational status of the HBV genome[4]. There is a higher occurrence of HBV genotypes B and C, and an increasing prevalence of HBsAg serostatus in China. Genotype C seems to have a stronger causative effect than genotype B on HCC development[4,14]. Therefore, it is hypothesized that pathogenicity may be associated with the HBV genotype.

HBV GENOME

HBV belongs to the *hepadnaviridae* family of enveloped viruses and has an incomplete double-stranded DNA genome of 3.2 kb (Figure 1). The double-stranded HBV genome consists of four open reading frames (ORFs): PreS1/PreS2/S ORF encodes the three large-, middle-, and small HBsAg proteins; C ORF encodes the hepatitis core protein (HBcAg) and HBeAg, a soluble small molecular weight protein produced by the viral core protein gene (PreC/C region) with an alternative start codon and post-translational modification[15]; P ORF encodes the four viral polymerases that consists of the RNase H, viral DNA polymerase/reverse transcriptase, spacer, and terminal protein; X ORF encodes the multi-functional non-structural hepatitis X protein, which can function as a transcriptional transactivator and promote HBV replication (Figure 1)[4,16,17]. The effects of HBV surface proteins and HBx protein on tumorigenesis have been widely studied but their molecular mechanisms remain elusive.

HBV PreS1/S2/S ORF

Three main HBsAg proteins are synthesized by three individual promoters and start sites. The PreS1 promoter drives the transcription of PreS1/S2/S ORF to form the large protein (L); the PreS2/S promoter drives the transcription of PreS2/S ORF to form the middle protein (M); while transcription of S ORF only forms the small protein (S) (Figure 1). All these HBsAg proteins have the same carboxyl (C)-terminus but different amino (N)-terminal extensions: the S protein forms the HBsAg with only 226 amino acids (aa), the M protein has an additional 55 aa N-terminal extension, while the L protein has a further N-terminal extension of 108, 118, or 119 aa, depending on the HBV genotype[5,18,19]. A central major hydrophilic region formed by residues 103-173 is exposed to the surface of the viral particles[5]. HBsAg is the main target for viral neutralization by either natural or vaccine-induced anti-HBs antibodies[5,18]. The PreS1 ORF plays a key role in HBV infectivity through its role in nucleocapsid binding during virus envelopment and in receptor binding during hepatocyte infection[18,20,21].

The PreS1 and PreS2 ORFs of HBV can initiate host immune responses as both contain several epitopes for B cell and T cell recognition. Therefore, mutations in these two ORFs can lead to a host immune response defect, resulting in chronic HBV infection[4,22]. Numerous clinical studies have shown deletions in the PreS1 3'-end and PreS2 5'-end are frequently found in chronic HBV infected patients[22-25]. Additional types of mutations that can occur in the PreS ORFs include deletions, insertions and substitutions. Importantly, presence of these mutations has been found to correlate closely with HCC tumorigenesis[4,22]. Deletion mutations that commonly occur can be classified into four categories: PreS1 start codon deletion, internal deletion of PreS1, PreS2 start codon deletion, and internal deletion of PreS2[23]. Generally, the PreS deletion sites are commonly located at the PreS1 and PreS2 region of the HBV genome (Figure 2)[23,25]. In addition, insertions and substitutions have also been reported in chronic HBV infected patient samples[25]. Most neutralizing antibodies target the immunodominant "a" determinant region that is formed by residues 124-147, whereas glycine to arginine substitution at position 145 (G145R) in the "a" determinant region of the S ORF occurs commonly in immune evasive mutant variants[5,18,26]. Other mutations occurring outside the "a" determinant region but within the S ORF, such as the P120S/T mutation, can also exhibit immune evasive effects. These mutant variants are associated with the clinical detection of HBV DNA in patient serum despite the

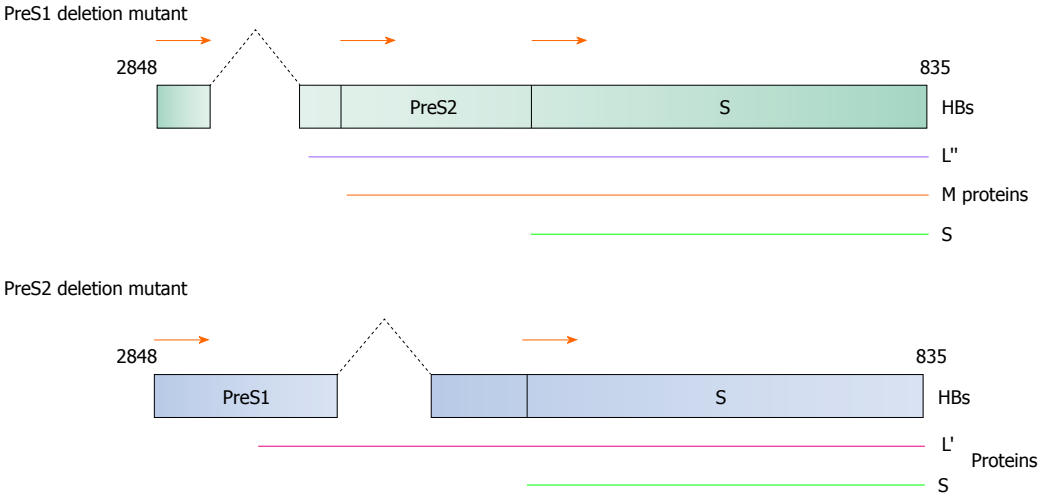

Figure 2 Diagrammatical representation of the PreS1/S2 deletion mutants. (Top) 3'-end PreS1 deletion mutant and (Bottom) 5'-end PreS2 deletion mutant are two common mutation forms found in HBV-induced HCC patients. Both mutants produce truncated large surface proteins (L' and L"). M: Middle protein; S: Small protein. HBs gene nucleotide position 2848 to 835 as shown in Figure 1.

absence of detectable HBsAg[5]. Substitution mutations can result in premature termination during gene transcription, resulting in the production of truncated HBsAg. These truncated HBsAgs can accumulate in the hepatic endoplasmic reticulum (ER), increase oxidative stress of the cell and eventually accelerate liver cell damage[23,25].

PreS deletion mutants can induce liver tumor formation by (1) altering the PreS1 mRNA to PreS2/S mRNA ratio; (2) inducing ER oxidative stress; and (3) allowing escape from host immune system surveillance, whereas the 3'-end deletion in the PreS2 domain and removal of the CCAAT element in the S promoter domain is also thought to reduce transcription of the 2.1 kb PreS2/S mRNA[23]. The CCAAT element increases the transcription of PreS2/S ORF and decrease transcription of PreS1 ORF, thus the deletion of this CCAAT element would alter the PreS1 mRNA to PreS2/S mRNA ratio. This decrease in transcription of PreS2/S ORF therefore reduces synthesis of S protein and eliminates synthesis of M protein. Normally, L protein can only be secreted as sub-viral particles or mature virions by forming a complex with the M and S proteins. Insufficient amounts of both M and S proteins would therefore result in the accumulation of L protein in hepatic ER that induces oxidative stress by generating high levels of reactive oxygen species (ROSs) that can cause oxidative DNA damage, induce genetic mutations, and ultimately lead to HCC development[23,27].

PreS deletion mutants have also been implicated in the prevention of apoptosis in hepatocytes by activating the nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 (NFKB1) and v-akt murine thymoma viral oncogene homolog 1 (AKT1)/mechanistic target of rapamycin (serine/threonine kinase) (MTOR) signaling pathways by upregulating the prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2) and vascular endothelial growth factor (VEGF) protein production, respectively[23,27]. In addition, hepatocytes transfected with the PreS2 deletion mutant can also evade the host immune system surveillance due to altered epitopes sites (b10, t5, t6) for B-cell and T-cell recognition and/or reduction in binding affinity of the viral proteins to major histocompatibility complex I molecule[23,27,28].

X ORF

The X ORF encodes the multifunctional HBx protein consisting of 154 aa[16,29]. The genetic mechanism(s) by which HBx induces and/or contributes to HCC development is still not well understood. However, it has been shown that HBx protein plays a critical role in hepatocyte transformation in three ways: (1) changing the epigenetic status; (2) inducing genomic instability; and (3) modulating signaling pathways[13,30].

HBx can change the epigenetic status of hepatocytes, leading to the inactivation of host tumor suppressor genes and/or activation of host oncogenes through induction of various DNA methyltransferases[30,31]. HBx is able to bind to histone acetyltransferase complex and CREB binding protein, promoting transactivation reactions and leading to histone hyperacetylation[13,29-31]. In addition, HBx can promote the production of H3-K4-specific methyltransferase by upregulating SET and MYND domain containing 3 (*SMYD3*) gene expression that increases H3 lysine K4 methylation[31]. HBx can recruit the binding of DNA (cytosine-5-)-methyl-transferase 1 and 3A (DNMT1 and DNMT3A) onto tumor suppressor genes and alter their methylation status and expression level. Conversely, HBx can also inhibit the binding of DNMT3A to promoters, releasing the hypomethylated status of tumor-promoting genes and inducing their expression[31]. *DNMT3A* has been shown to be associated with hepatocarcinogenesis, as a higher expression level of *DNMT3A* has been reported

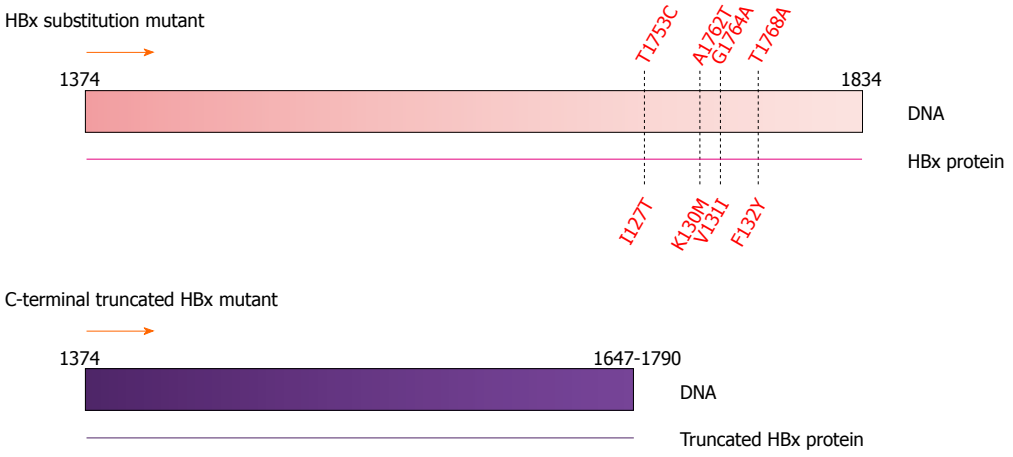

Figure 3 Common mutations in the HBx gene. Substitution mutations at nucleotide positions T1753C, A1762T, G1764A and T1768A; and C-terminal truncations ranging from nucleotide position 1647 to 1790 observed in human HCC tumor samples[43]. HBx gene nucleotide position 1374 to 1834 as shown in Figure 1.

in HCC patients[30]. The transcriptional transactivation activity of HBx includes the upregulation of *DNMT1* and *DNMT3A*, which induces cytosine-guanine dinucleotide (CpG) island methylation at the carbon-5 position of cytosine. This *DNMT1* and *DNMT3A* upregulation prevents binding of transcription factors and RNA polymerase Ⅱ complexes to tumor suppressor genes, such as cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*), resulting in their inactivation[30,31]. In addition, HBx can also promote binding of histone deacetylases to tumor suppressor genes to suppress their transcription[31].

In addition to inducing epigenetic changes, HBx is also believed to enhance genomic instability. HBx has been associated with the inactivation of various tumor suppressor genes such as tumor protein p53 (*TP53*), retinoblastoma 1 (*RB1*), and axin 1 (*AXIN1*). HBx inhibits major DNA repair pathways by the interaction with tumor suppressor proteins in these pathways, leading to enhanced genomic instability from the accumulation of mutations and deletions[32-35]. HBx can also increase angiogenesis by upregulating *VEGF* transcription and stabilizing hypoxia inducible factor 1, alpha subunit [basic helix-loop-helix transcription factor (*HIF1A*)][29]. Recently, several *in vivo* research studies have demonstrated HBx overexpression upregulates catenin (cadherin-associated protein), beta 1, 88kDa (*CTNNB1*) in the important canonical wingless-type MMTV integration site family (WNT)/CTNNB1 signaling pathway implicated in hepatocarcinogenesis[29,36,37].

Four common mutation sites have been identified in the HBx ORF: nucleotide position T1753C (I127T amino acid substitution), A1762T (K130M amino acid substitution), G1764A (V131I amino acid substitution) and T1768A (F132Y amino acid substitution)[38] (Figure 3). The combinatory effects of these mutations have been studied *in vitro* using the human liver cell line (CCL13) where both K130M and V131I mutations had the potential to induce cell proliferation[38,39].

Another form of HBx mutation is the C-terminal truncation (Figure 3). The role of HBx in HBV replication has been carefully studied in HepG2 and Huh7 cell lines[16,40,41]. HBx contains two active domains: a negative regulatory domain in the N-terminus and a transactivation or co-activation domain in the C-terminus that can transactivate viral and cellular promoters[16]. *In vitro* experiments have shown 52 to 65 aa and 88 to 154 aa in the C-terminus of HBx are necessary for its transactivation activity, cell cycle regulation, and HBx stability[16,40,41]. Upon integration of the HBV genome into the host genome, the 3'-end of HBx is often deleted and produces the C-terminal truncated form of HBx[42,43]. Clinically, C-terminal truncated HBx has been commonly detected in tumors of HCC patients[42,44,45]. Importantly, this C-terminal truncated HBx has been shown to promote tumor cell proliferation and metastasis compared to full-length HBx[42,44,46]. Furthermore, C-terminal truncated HBx has shown to abrogate the growth suppression and apoptotic effect of full-length HBx in cell lines[42-44,46]. However, the genetic mechanism(s) of how this C-terminal truncated HBx affects the tumorigenicity of HBV still remains unclear.

MECHANISMS OF HBV-INDUCED HCC

Increasing evidence has shown HBV infection plays an important role in the development of HCC. Two major mechanisms involved in HBV-associated HCC pathogenesis are: (1) integration of the viral genome into the host chromosome; and (2) the expression of the *trans*-activating factors derived from the HBV genome[13,15,29,47]. Although, during the normal lifecycle of HBV, the viral genome present in the host nucleus is a covalently closed circular DNA (cccDNA) (Figure 4), about 80% of HBV-related HCC are found to have the HBV genome integrated into the host chromosomes[13]. The integration of the viral genome into the host DNA can disrupt and/or promote cellular gene expression

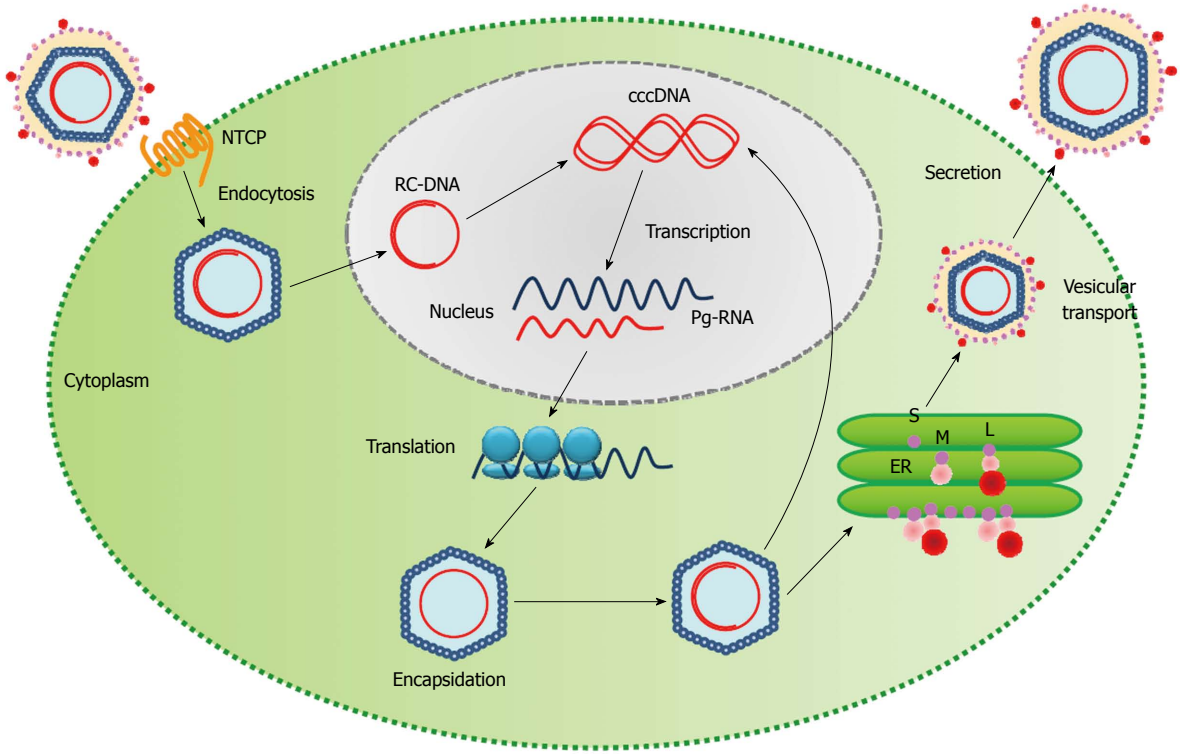

Figure 4 Life cycle of the hepatitis B virus. HBV enters the hepatocyte and is uncoated in the cytoplasm. The relaxed-circular viral DNA (RC-DNA) is released from the nucleocapsid into the host nucleus and is converted into covalently closed circular DNA (cccDNA). The cccDNA acts as a template for transcription of pregenomic virion RNAs (Pg-RNA). Pg-RNA is then transferred into the cytoplasm, bound by viral polymerase and encapsidated. Inside the nucleocapsid, the Pg-RNA is reverse transcribed into RC-DNA, where either the RC-DNA can be released back into the host nucleus for amplification of viral DNA or the entire nucleocapsid can be transferred into the endoplasmic reticulum (ER) for viral surface protein coating and ultimately released from the cell[13]. S: Small surface protein; M: Middle surface protein; L: Large surface protein; HBV: Hepatitis B viral.

involved with cell growth and differentiation[47]. In addition, the expression of *trans*-activating factors derived from the expression of viral DNA may influence intracellular signaling pathways and affect host gene expression[47]. Elevated levels of truncated PreS2/S, HBx, and hepatitis B spliced protein have been found in infected tumor tissue[47]. HBx has been shown to play a role in pleiotropic functions and to be involved in the malignant transformation of chronically-infected liver cells[47]. HBx can induce cell proliferation, oxidative stress, and host DNA damage and ultimately contribute to HCC tumorigenesis[13,15]. Recent studies have found HBx can activate *CTNNB1* expression and inactivate *TP53*[15,37]. The activation of the WNT/CTNNB1 signaling pathway is vital in the genetic evolution of HCC[48]. Elevated levels of truncated surface proteins have also been found in HCC patients chronically infected with HBV.

MOST FREQUENTLY MUTATED GENES IN HBV-ASSOCIATED HCC

The relationship of somatic mutation of telomerase reverse transcriptase (*TERT*) to different types of cancer has been highly reported. Recently, the relationship between *TERT* and HCC is becoming more obvious: the *TERT* promoter was shown to be mutated in 54% of 469 HCC cases and in 37% of HBV-positive cases, where the mutation site was most commonly located 124 bp upstream the *TERT* start codon[49]. Moreover, there was a 22% focal amplification rate of *TERT* in HBV-positive samples[49]. The combined *TERT* promoter mutation and focal amplification causes higher *TERT* expression in HCC[49]. Apart from *TERT*, recent exome sequencing of HCC tumors has identified at least 30 significant putative HCC driver genes involved in 11 signaling pathways: WNT/CTNNB1, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT/MTOR, TERT, TP53/cell cycle, hepatic differentiation, epigenetic regulation, chromatin remodeling, oxidative stress, IL6/Janus kinase (JAK)-STAT3 and TGFB[49,50]. Amongst these putative driver genes, somatic mutations and copy number changes in *CTNNB1*, *TP53*, *AT rich interactive domain 1A* (*SWI-like*) (*ARID1A*) and *AXIN1* were significantly altered in 10% of HCC patient samples[49,50]. *TP53* mutations are common in most cancer types, so it is not surprising that similar *TP53* mutation frequencies in HBV-related HCC have been widely reported by numerous studies[37,49-51]. Furthermore, 16% of the *TP53* mutations identified in genotype B HBV-related HCC were found at the R249S site, usually associated with aflatoxin-induced HCC[51-53].

THE NEED FOR MOUSE MODELS OF HBV-INDUCED HCC

Although a few mechanisms by which HBV may promote HCC have been identified, they need to be better characterized in order for the understanding of the genetic mechanisms of HBV-induced HCC to be complete. While it is promising that several common mutations driving HCC in context of HBV infection have been identified, there are many more mutations present in HBV-associated liver tumors that may be important in driving tumor phenotypes[54,55]. Of the many mutations found in HBV-associated liver tumors, the drivers need to be identified and characterized. Mouse models may provide an effective tool for this.

FORWARD GENETIC SCREENS FOR DRIVERS OF HCC USING TRANSPOSON INSERTIONAL MUTAGENESIS

The discovery of inducing lymphoma and mammary cancer formation in mouse models through retroviral insertional mutagenesis by murine leukemia virus or mouse mammary tumor virus has accelerated the speed of identifying driver mutations within these tumor types[56]. However, the application of retroviral insertional mutagenesis has been limited to these specific cancers. Therefore, it may be more valuable to apply an insertional mutagenesis method that is applicable in oncogenomic studies for more human cancer types.

Transposons are such genetic tools that can be used for insertional mutagenesis in multiple tissues in most mammalian species. One transposon that has been successfully used for both forward and reverse genetic interogation of the oncogenome is Sleeping Beauty (*SB*)[57]. The synthetic *SB* transposon belongs to the *Tc1*/mariner family of class Ⅱ transposable elements[57]. The *SB* transposon system consists of two components: a transposon and transposase. The transposon can be any DNA sequence flanked by inverted repeat/direct terminal repeat (IR/DR) sequences. The transposase binds to the IR/DR sequences and mediates the excision and reintegration of the transposon from one locus to another in a "cut-and-paste" manner[57]. The target integration site for *SB* is a TA-dinucleotide pair and, with approximately 300 million sites in the mouse genome, allows for ample sites for insertional mutagenesis[58,59]. The process of transposition or mobilization is relatively random, although "local hopping" may occur[60-62].

Tissue-specific mutagenesis with the *SB* transposon system has been made possible with the use of a conditional system, which has allowed for transposon insertional mutagenesis in mice to recapitulate several important human cancers for genetic analyses, including liver, gastrointestinal tract, skin, blood, bone, prostate and nervous system[63-75]. In this review we focus on the use of the conditional *SB* insertional mutagenesis system in several forward genetic screens for HCC candidate genes[63-67,75]. Briefly, mice carrying the following transgenes were generated for each of these forward genetic screens for liver cancer genes: conditional *SB* transposase transgene, mutagenic transposon, hepatocyte-specific *Cre* recombinase and predisposed genetic background[63,66]. The *SB* transposase (*SB11*) carrying a floxed-stop (lsl) cassette knocked into the mouse endogenous *Rosa26* locus can only be activated by a tissue-specific *Cre* recombinase, allowing for expression and mobilization of transposons exclusively in hepatocytes[64]. The mutagenic transposon, T2/Onc, consists of splice acceptor/polyadenylation (SA/pA) sequences in both orientations and a murine stem cell virus (*MSCV*) long terminal repeat (LTR) containing promoter/enhancer elements followed by a splice donor that can facilitate splicing of transcripts initiated in the *MSCV* into downstream endogenous exons[68,74]. The mutagenic transposon was designed to allow for both gain-of-function and loss-of-function mutational activity when integrated into the host genome. When the mutagenic transposon is inserted in a tumor suppressor gene, normal splicing event will be disrupted by the SA/pA elements. Alternatively, misexpression of a proto-oncogene by the *MSCV* LTR element will occur if the mutagenic transposon is inserted within or near these genes[68,74].

Since *TP53* is commonly mutated in HBV-related HCC[51-53], and HBx has been found to inactivate *TP53*[76-78], studying the genetic drivers of HCC in context of transformation related protein 53 (*Trp53*) mutation in a mouse model may shed light on the genetic alterations required for HBV-induced HCC development. Using the conditional transposon system together with a conditional dominant negative *Trp53* transgene as a predisposed genetic background, 19 highly significant common insertional sites (CISs) for HCC-associated genes were identified[66]. The three most significant signaling/disease functional annotations were identified through analyzing CIS genes by Ingenuity Pathway Analysis (IPA): post-translational modification, cancer, and tumor morphology. Epidermal growth factor receptor (*EGFR*), *HIF1A*, mitogen-activated protein kinase kinase 4 (*MAP2K4*), MET proto-oncogene, receptor tyrosine kinase (*MET*), p21 protein (*Cdc42/Rac*)-activated kinase 4 (*PAK4*), vaccinia related kinase 2 (*VRK2*), transient receptor potential cation channel, subfamily M, member 7 (*TRPM7*) and TAO kinase 3 (*TAOK3*) have been shown to be involved in tumor formation and apoptsis[66]. Nuclear factor I/B (NFIB) and HIF1A network pathways were also identified by IPA and have been implicated in the transduction of phosphorylation-signaling cascades from *EGFR*[66]. *PAK4*, *NFIB*, *TAOK3*, *EGFR*, *MET*, *MAP2K4*, *HIF1A*, ubiquitin-conjugating enzyme E2H (*UBE2H*), and QKI,

Table 1 Hepatocellular carcinoma signaling pathways identified by Sleeping Beauty transposon insertional mutagenesis system[63,66]

Pathways	Wnt/Ctnnb1	Trp53	Pi3k-Akt-Mtor	Mapk	Hippo	Tgfb-Bmp	Il6-Stat3	Tnf-Akt[1]
Genes involved in corresponding pathways	*Lrp1*	*Prkag2*	*Insr*	*Egfr*	*Fat1*	*Acvr1*	*Il6st (gp130)*	*Tnf*
	Lrp5	*Ywhaz*	*Igf1*	*Met*	*Wwc1*	*Acvr2a*	*Jak1*	*Egfr*
	Lrp6	*Crebbp*	*Pten*	*Grb2*	*Taok1*	*Bmp1*	*Stat3*	*Met*
	Gsk3b	*Mdm2*	*Pik3ca*	*Sos1*	*Taok2*	*Bmpr1a*		*Pak4*
	Axin1	*Usp10*	*Pik3r1*	*Sos2*	*Taok3*	*Sar1a*		*Nfib*
	Apc	*Usp7*	*Pik3c2a*	*Kras*	*Mobkl2b*	*Tab2*		*Taok3*
	Ctnnb1	*Pias1*	*Pik3ap1*	*Raf1*	*Mobkl1a*	*Tab3*		*Map2k4*
	Tcf7l2	*Trp53inp1*	*Akt2*	*Map3k1*	*Sav1*	*Smad3*		*Akt*
	Csnk1a1	*Ppp2r1a*	*Rps6kb1*	*Map3k2*	*Lats1*	*Smad2*		*Hif1a*
	Csnk1d	*Ppp2r2a*	*Foxo1*	*Map2k1*	*Yap1*	*Smad1*		*Ube2h*
	Csnk1g1	*Ppp2r2d*		*Mapk1*	*Tead1*	*Smad4*		*Qk*
	Csnk1g3	*Ppp2cb*				*Smad5*		*Pi3k*
	Tnks	*Ppp2r5e*						*Jnk*
	Tnks2	*Trp53*						

[1]Indicates a network.

KH domain containing, RNA binding (*QKI*) were found to potentially interact with tumor necrosis factor (*TNF*), inducing tyrosine phosphorylation and internalization of *EGFR* that might activate the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1) pathway that regulates apoptosis during liver tumor formation (Table 1)[66]. In addition, a high frequency of mutagenic transposon insertions were found in intron 24 of the *Egfr* gene. This insertion results in the production of C-terminal truncated Egfr protein, which transphosphorylates the tyrosine sites of v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (Erbb2) and activates other signaling pathways that contribute to HCC tumorigenesis[66]. In addition, this study also revealed 3 strong candidate genes (*UBE2H, QKI, MAP2K4*) associated with HCC. *QKI* and *MAP2K4* have a significant decrease in DNA copy number and mRNA level and a high proportion of deletion mutations in human HCC samples, suggesting that both are putative HCC tumor-suppressor genes[66,79,80]. The significant increase in DNA copy number, mRNA level, and high mRNA up-regulation rate of *UBE2H* indicate that it is a putative HCC tumor-promoting gene[66,79,80].

Incorporating the *SB* transposon system with a commonly dysregulated human malignancy gene, v-myc avian myelocytomatosis viral oncogene homolog (*MYC*), 18 CISs in early-developing liver tumors were identified[67]. It was shown that zinc finger protein, X-linked (*Zfx*) plays a tumor suppressor role in liver tumorigenesis, while nuclear receptor coactivator 2 (*Ncoa2*) and dystrobrevin, beta (*Dtnb*) function as putative tumor-suppressors[67]. In addition, expression of *NCOA2* and its target gene glucose-6-phosphatase, catalytic subunit (*G6PC*) were significantly reduced in human tumor samples, and this reduction was strongly associated with the low survival rates of HCC patients[67].

Using the *SB* transposon insertional mutagenesis mouse model incorporating the HBsAg transgene in a forward genetic screen, over 2000 candidate drivers of HBV-associated HCC were identified[63]. In this study, 21 genes with highly significant sequencing read counts and frequencies of occurrence were identified to be candidate HCC genes[63]. Of these candidate genes, 4 genes were identified as putative tumor suppressor genes: adenosine kinase (*Adk*), dihydropyrimidine dehydrogenase (*Dpyd*), lysine (*K*)-specific methyltransferase 2E (*Kmt2e*) and nuclear factor I/A (*Nfia*); 6 genes were found to have tumor suppressing effects in HCC: *Arid1a*, *Gsk3b*, IG motif containing GTPase activating protein 2 (*Iqgap2*), membrane associated guanylate kinase, WW and PDZ domain containing 1 (*Magi1*), phosphatase and tensin homolog (*Pten*) and salvador homolog 1 (*Sav1*); 2 genes were found to be involved in hepatocyte differentiation and maturation: zinc finger and BTB domain containing 20 (*Zbtb20*) and ankyrin repeat domain 17 (*Ankrd17*); 6 genes were found to regulate hepatic metabolism: *Pten*, glycogen synthase kinase 3 beta (*Gsk3b*), growth hormone receptor (*Ghr*), *Adk*, *Zbtb20*, and *Dpyd*; and 7 genes were found to be associated with HCC transcription modulation: *Kmt2e*, SET domain containing 2 (*Setd2*), WW domain containing adaptor with coiled-coil (*Wac*), *Arid1a*, *Nfia*, staphylococcal nuclease and tudor domain containing 1 (*Snd1*), and *Zbtb20*[63]. Through CIS gene annotation enrichment analysis, it was found that HBsAg CIS genes drive HCC through conserved cancer signaling pathways, including Wnt/Ctnnb1, Trp53, Pi3k-Akt-Mtor, Mapk, Hippo, transforming growth factor beta (Tgfb)-bone morphogenic protein (Bmp) and Il6-signal transducer and activator of transcription 3 (Stat3) (Table 1)[63]. Moreover, it was revealed that the majority of the HCC CIS genes were involved in cellular metabolic processes[63]. It was suggested that disruption of glycolytic and glutaminolytic pathways provided bioenergetics, biosynthesis and redox regulation benefits for tumor cell division, thus inhibiting

Figure 5 Word cloud of frequent CIS genes from three separate studies using *SB* transposon forward genetic screening with different genetic backgrounds[63,65,67]. The roles of these frequent CIS gene human orthologs were analyzed using TCGA database. The font size indicates the frequency of genetic alteration reported in human HCC samples. Color of the font indicates the copy number change or expression profile of the gene: red, amplification; blue, deletion; tomato, tends to upregulation; purple, tends to downregulation; yellow, frequently found in all three studies.

glycolysis and/or glutaminolysis might impede liver tumor formation or progression[63]. Genetic alterations, copy number changes, and expression levels of the human orthologs of CIS genes from three separate *SB* transposon forward genetic screens with different genetic backgrounds were analyzed in TCGA database and presented as a word cloud (Wordle) to view the degree of genetic alteration reported in clinical samples (Figure 5)[63,65,67].

REVERSE GENETIC VALIDATION USING RAPID TRANSPOSON-BASED MOUSE MODELS

The *SB* transposon system can also be used in a reverse genetic manner to introduce tumorigenic genes into mouse hepatocytes for stable expression when delivered on transposon plasmids to the mouse liver by hydrodynamic tail vein injection[37,59,65,66,75,81]. Hydrodynamic injection is a rapid and high-volume infusion of naked plasmid DNA into the tail vein. It is an effective method for *in vivo* gene delivery, in which about 40% of the hepatocytes in a test animal take up the transgene and express > 95% of the transgene after hydrodynamic injection[59]. The mechanism of DNA uptake is still poorly understood, but it is suggested that the injected high-volume of DNA solution enters the inferior vena cava and causes over-stretching of myocardial fibers, induces cardiac congestion, resulting in delivery of injected solution into liver[82]. In addition to its use in mice, this method can also be used to transfer naked plasmid DNA into porcine and rabbit livers and into muscles of larger animals[59,82]. The sporadic expression of target genes mimics a more realistic situation in human liver cancer than other conventional conditional transgenic models.

A powerful method to test genes' oncogenic roles in the liver employs this gene delivery system to deliver tumorigenic genes to the livers of fumarylacetoacetate hydrolase (*Fah*)-deficient/*Rosa26*-SB11 (*Fah*$^{-/-}$/*SB*$^{+/-}$) transgenic mice[37,59,65,66,75,81,83]. This is a selective model allowing for rapid generation of mice in which nearly all hepatocytes express the delivered transgenes. *Fah*-deficient mice have a defect in the last step of the tyrosine catabolic pathway in which fumarylacetoacetate is hydrolyzed to acetoacetate and fumarate, similar to the human hereditary tyrosinemia type I disease[84]. These mice must be maintained on nitisinone in the drinking water, which blocks this pathway upstream of fumarylacetoacetate production[84]. Oncogenes to be tested are co-delivered on transposon plasmids with a *Fah* rescue cDNA, and nitisinone is removed after gene delivery. Nitisinone removal causes *Fah*-deficient hepatocytes to die, and the liver is regenerated by hepatocytes that stably express the delivered transgenes[37,59,65,66,75,81]. This system has been used in a reverse genetic manner to introduce tumorigenic genes into the livers of *Fah*$^{-/-}$/*SB*$^{+/-}$ transgenic mice by hydrodynamic tail vein injection for validation in several studies[37,59,65,66,75,81].

SB-mediated gene delivery to *Fah*$^{-/-}$/*SB*$^{+/-}$ transgenic mice by hydrodynamic tail vein injection has been used to study the role of HBx in promoting liver cancer[37]. In this study, a transposon vector containing the HBx gene and *Fah* cDNA was delivered with and without a transposon vector containing a short hairpin RNA directed against *Trp53* (shp53). HBx expression

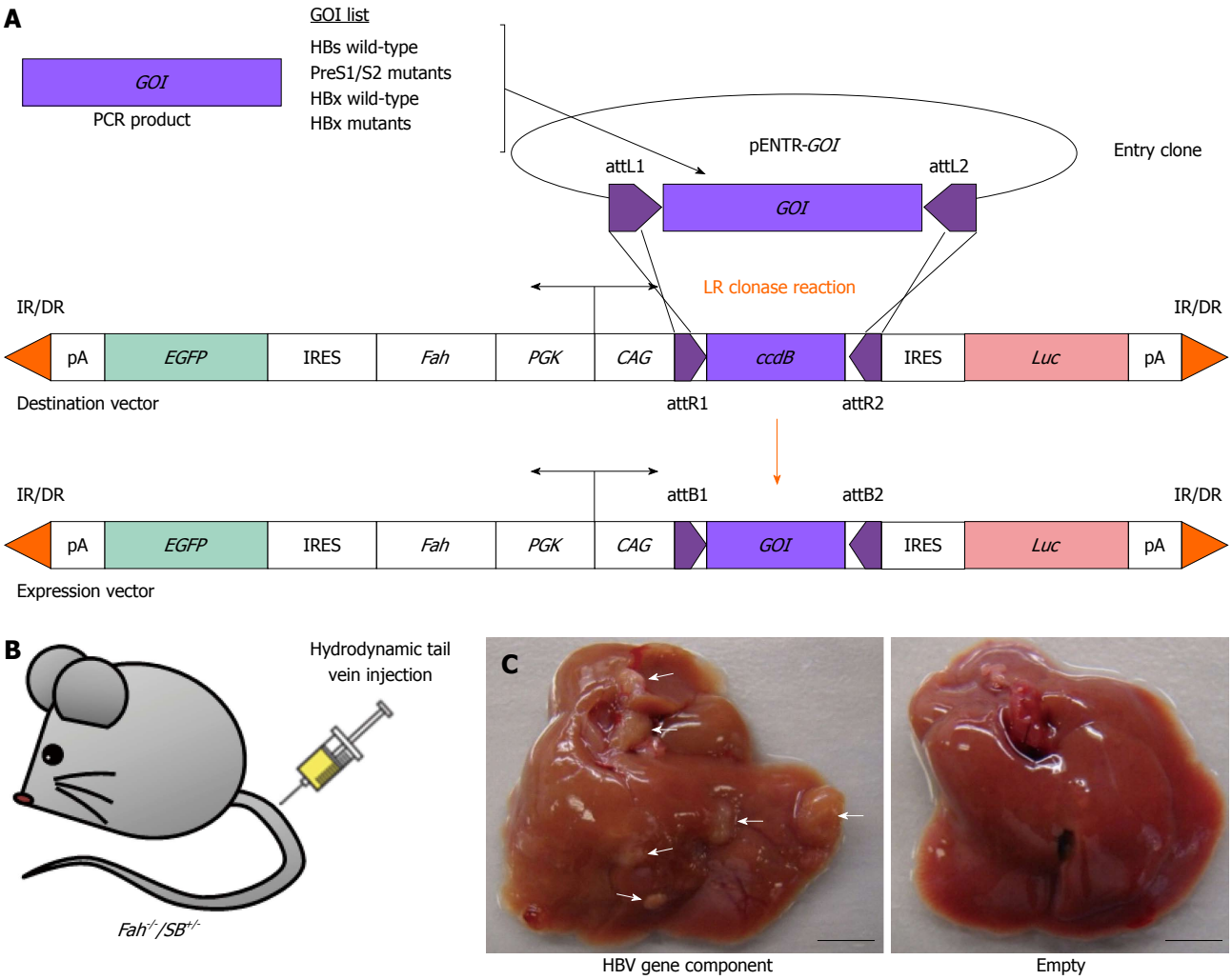

Figure 6 Reverse genetic screening of individual HBV gene components using the *Fah*$^{-/-}$/*SB*$^{+/-}$ transgenic mouse model. A: Construction of gene delivery expression vectors that carry various individual HBV gene-of-interest (*GOI*) components by PCR amplification and insertion of *GOI* into an entry clone (pENTR) and followed by LR clonase reaction (Life Technologies); B: Hydrodynamic tail vein injection of gene delivery expression vector into *Fah*$^{-/-}$/*SB*$^{+/-}$ transgenic mouse; C: Liver tumors indicated by white arrowheads observed in *Fah*$^{-/-}$/*SB*$^{+/-}$ mouse injected with a HBV gene component (left) after 160-d post-hydrodynamic injection. *Fah*$^{-/-}$/*SB*$^{+/-}$ transgenic mouse injected with an empty gene delivery expression vector displayed normal liver morphology (right). *EGFP*: Enhanced green fluorescent protein gene; *Luc*: Luciferase gene; IR/DR: Inverted repeat/direct repeat sequences for Sleeping Beauty transposase binding and mobilization; pA: Polyadenalytion signal; IRES: Internal ribosome entry site; *PGK*: Phosphoglycerate kinase 1 promoter; *CAG*: Cytomegalovirus enhancer fused to the chicken β-actin promoter; attL/R/B sites: Recombination sites used by the Gateway cloning system (Life Technologies); *ccdB*: Topoisomerase poison from *Escherichia coli*. Scale bar: 0.5 cm.

activated *Ctnnb1* expression, and HBx cooperated with shp53 to induce the formation of hyperplastic nodules. This study showed this method could be used to model liver cancer driven by HBV gene components and elucidate mechanisms by which they may promote cancer.

We are currently attempting to use this method to test the roles of other HBV genes in driving liver cancer. Using the Gateway cloning system (Life Technologies), we have constructed transposon plasmids with various individual HBV gene components for tumorigenic analyses in livers of *Fah*$^{-/-}$/*SB*$^{+/-}$ transgenic mice. Hepatocytes that are transgenic for both the *Fah* cDNA and HBV gene-of-interest will repopulate the *Fah*-deficient liver, mimicking disease progression. Preliminary unpublished studies have yielded promising development of tumors in certain HBV gene-injected mice compared with empty vector controls (Figure 6). These tumors will be further interrogated for their genetic information such as gene expression profiles and/or pathway analyses. It is envisaged that genetic information from such *in vivo* studies will provide insight into underlying genetic mechanisms of HBV-induced HCC.

SB-mediated gene delivery for reverse genetic studies in mice, in addition to its use in studying the oncogenic roles of viral genes, could be used to rapidly validate candidate genetic drivers of HBV-associated HCC discovered in forward genetic screens as described above or genes found to be commonly altered in human HBV-associated HCC. Candidate drivers could be tested in context of expression of HBV viral genes using this system. In addition, hydrodynamic delivery of plasmid DNA expressing

HBV genome components has been used to model HBV infection in mice[85-87]. Candidate HBV-associated HCC drivers could be added on separate plasmids to test their roles in HCC in context of a model of HBV infection *in vivo*.

DISCUSSION

Based on the limited existing treatments and the poor prognosis of HCC, continuous efforts should be put into uncovering the mechanisms of drug resistance and tumor progression and on the development of biomarkers for more sensitive diagnosis and as molecular targets for new therapies. HBV infection is a major risk factor for HCC development; understanding its interactions with cancer-driving signaling pathways and its contributions to tumor initiation, promotion and progression will be critical for developing biomarkers for diagnosis and therapy.

In addition, recent exome sequencing of HCC patient samples have identified multiple cellular signaling pathways, including WNT/CTNNB1, MAPK, PI3K/AKT/MTOR, TERT, TP53/cell cycle, hepatic differentiation, epigenetic regulation, chromatin remodeling, oxidative stress, IL6/JAK-STAT3 and TGFB[49,50]. In addition, numerous significant HCC driver genes, such as *TERT, TP53, CTNNB1, AXIN1* and *ARID1A* have also been identified[49,50]. Additionally, *TP53* mutation was frequently found in HBV-related HCC patient samples[50]. A large number of components are involved in each of these pathways, many of which may pose effects on the tumor initiation, promotion and progression, which challenges the screening of potential therapeutic drugs for treatment. Insertional mutagenesis by the *SB* transposon system is a powerful tool for studying HBV-induced HCC through forward genetic screens, and *SB*-mediated gene delivery is a powerful tool for reverse genetic studies. *Snd1,* an oncogene that promotes HCC angiogenesis, was a recurrent CIS gene in liver tumor samples from various studies using the *SB* transposon system forward genetic approach including a study done in the context of transgenic HBsAg expression[63,65-67]. Many other candidate genes likely to play a role in promoting HBV-associated HCC have been identified in these studies. There is also ample data on genes commonly mutated in HBV-associated liver tumors, and genes whose expression or function may be altered by HBV proteins. We have developed a unique rapid *in vivo* model to validate candidate liver cancer genes that can be used to study potential drivers of HBV-associated HCC. It relies on the use of the *Fah*-deficient mouse that is transgenic for the *SB* transposase gene ($Fah^{-/-}/SB^{+/-}$)[81,84]. As described earlier, candidate genes can be easily cloned into transposon-based delivery gene vectors and introduced specifically into the livers of these mice using hydrodynamic tail vein injection[59]. This system can be used to study both cellular and viral genes. Importantly, these models could be used in pre-clinical trials to test novel therapeutic drugs. Therefore, we propose to use this rapid system to generate many genetic mouse models of HBV-induced HCC to further investigate the tumorigenic mechanisms. Currently, we have generated mouse models that recapitulate several molecular subclasses of human HCC: neuroblastoma RAS viral (*v-ras*) oncogene homolog (*NRAS*), *CTNNB1*, Poly7, and HBV-induced[37,65,81]. The heterogeneity and complexity of HBV-induced HCC has thus far precluded the full understanding of the genetic mechanisms associated with this deadly disease needed to develop effective treatments and preclinical models. We believe the *SB* transposon system may allow the discovery of drug targets and development of preclinical models desperately needed to advance this field.

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Hypoxia inducible factor in hepatocellular carcinoma: A therapeutic target

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Abstract

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed and deadly cancers worldwide; its incidence has been rising in the United States due to the increase in hepatitis C associated cirrhosis and the growing epidemic of obesity. There have been no effective therapeutic options in the advanced disease setting beyond sorafenib, a multi-targeted tyrosine kinase inhibitor that showed significant survival benefit. Because of this, there is an urgent need to search for novel pathways in sorafenib experienced patients. This review will focus on the role of hypoxia and hypoxia-inducible factor alpha (HIF-1α) in cancer development, specifically in HCC. We will discuss the biology of HIF-1α, the pathways with which it interacts, and the function of HIF-1α in HCC. Furthermore, we will review studies highlighting the relevance of HIF-1α in the clinical setting, as well as the pre-clinical data supporting its further investigation. Finally, we will conclude with a discussion of the potential role of a HIF-1α mRNA antagonist for the treatment of HCC, and hypothesize the ways in which such an inhibitor may be best utilized in the management of advanced HCC. Hypoxia plays a significant role in the development of HCC. HIF-1α is a key transcription factor involved in the hypoxic response of cancer cells. It activates transcription of genes responsible for angiogenesis, glucose metabolism, proliferation, invasion and metastasis in HCC. Its involvement in multiple, essential tumor pathways makes it an attractive potential therapeutic target in HCC.

Key words: Hypoxia; Hypoxia-inducible factor alpha; Hepatocellular carcinoma; Vascular endothelial growth factor

Core tip: Beyond sorafenib, limited systemic treatment options exist for the treatment of advanced hepa-

tocellular carcinoma (HCC). Hypoxia and hypoxia-inducible factor alpha (HIF-1α) have emerged as important factors in the development of HCC. This review focuses on the scientific background and pre-clinical data of HIF-1α and will conclude in a discussion of the clinical relevance of this transcription factor and its potential therapeutic role, particularly in combination with other therapies, in HCC. A phase IB clinical trial to investigate a HIF-1α mRNA antagonist in HCC patients who have failed first line systemic treatment is currently underway.

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INTRODUCTION

Incidence of hepatocellular carcinoma

Worldwide, hepatocellular carcinoma (HCC) remains the 5th and 7th most common cancer diagnosed in men and women, respectively; it is responsible for over 500000 deaths annually[1]. HCC typically occurs in the setting of chronic liver disease and cirrhosis, which are closely related to risk factors such as hepatitis B or C infection and alcohol abuse. In the United States, the incidence of HCC is rising, owing in large part to the increase in hepatitis C associated cirrhosis, and the latency that exists between timing of hepatitis C infection, cirrhosis, and ultimately development of HCC[2]. Although the recent successful treatment of hepatitis C has brought about excitement in the medical community, the potential for patients with preexisting, residual cirrhosis to develop HCC may increase due to prolonged lifespan after treatment of viral infection. Moreover, the rising incidence of obesity and its association with metabolic syndrome has paralleled the rise in HCC, with insulin resistance, increased tissue necrosis factor activity, and non-alcoholic steatosis all implicated as possible mechanisms of pathogenesis for HCC[3].

Current treatment options in HCC

For early stage disease, which represents only 15% of all patients with HCC, potentially curative therapeutic options include liver resection, transplantation, and ablation. Nevertheless, recurrence of disease or development of metastases commonly occurs in patients post-resection, with 50% of patients recurring within two years; the three-year survival rates post recurrence have been 10%-40%, depending on the stage of disease[4,5]. The majority of patients with HCC often present with advanced disease not amenable to cure or locoregional therapy, and only systemic therapy can be offered.

Systemic therapy for HCC

Considered chemotherapy-refractory, HCC has seen very few successful advances within the realm of systemic treatment. With the success of the randomized, phase Ⅲ SHARP trial by Llovet and colleagues in 2008, sorafenib, an oral multikinase inhibitor of the vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and Raf serine/threonine kinases, has established itself as the standard systemic treatment for advanced, unresectable HCC, based on an improvement in overall survival (OS) of nearly three months compared to placebo[6]. Since then, unfortunately, no agent has come to demonstrate a similar, significant survival advantage in the first-line setting. Moreover, despite numerous studies of various treatment options, no agent has proven beneficial as a standard second-line therapy. Therefore, investigation of novel and effective therapy in the second-line setting is urgently warranted.

Hypoxia, HIF-1, and cancer

Hypoxia, a reduction in tissue oxygen tension due to inadequate oxygen supply, has been implicated in pathways promoting tumor growth. Although hypoxia itself is toxic to cancer cells, it also appears to induce a series of adaptive, "pro-survival" changes in the tumor, which include a shift from aerobic to anaerobic metabolism, an increase in erythropoietin to promote rise in hemoglobin, and an increase in growth factors leading to angiogenesis[7]. Furthermore, hypoxia has been associated with resistance to chemotherapy and radiotherapy, and is closely related to poor clinical outcomes. Hypoxia-inducible factor (HIF-1) is an important transcription factor involved in the hypoxic response of cells, and functions in tumor development and progression. One of its target genes, vascular endothelial growth factor (VEGF), is one of the major components of angiogenesis and tumor proliferation. Among the subunits of HIF-1, HIF-1α has been implicated in cancer progression. This review will therefore focus on the scientific background of HIF-1α, its biology, existing pre-clinical data, and its potential role in the treatment of advanced HCC.

HIF-1: BIOLOGY AND SIGNIFICANCE

Structure of HIF-1

HIF-1 is a heterodimeric transcription factor which consists of the oxygen-sensitive HIF-1α and the constitutively expressed HIF-1β [also known as aryl hydrocarbon receptor nuclear translocator (ARNT)]. Both subunits contain basic helix-loop-helix (bHLH) motifs and PER-ARNT-SIM (PAS) domains needed for dimerization with hypoxia response elements (HRE) in the promoter region of target genes[8] (Figure 1).

HIF-1α degradation

The expression of HIF-1α is determined by both its

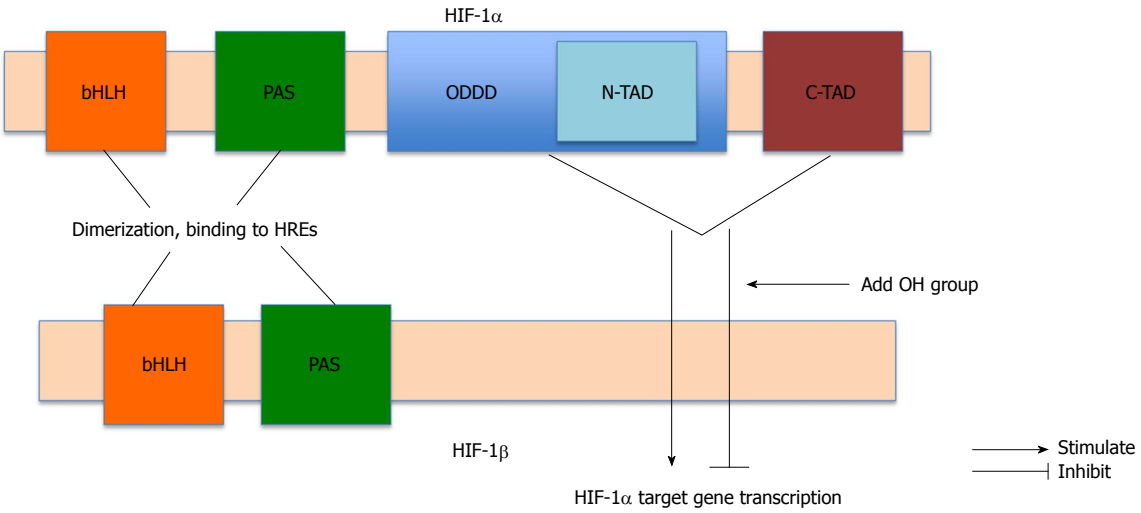

Figure 1 Structure of hypoxia-inducible factor-1. Hypoxia-inducible factor (HIF)-1 is a heterodimer comprising HIF-1α and HIF-1β. Both subunits contain basic helix-loop helix (bHLH) motifs and PER-ARNT-SIM (PAS) domains needed for dimerization and binding to hypoxia response elements (HREs) in the promoter region of target regions. HIF-1α contains two transactivation domains: the NH_2-terminal transactivation domain (N-TAD), located on the oxygen-dependent degradation domain (ODDD), and the carboxy-terminal transactivation domain (C-TAD). Both transactivation domains are essential for promoting HIF-1α target gene transcription. Hydroxylation of N-TAD and C-TAD of HIF-1α leads to inhibition of HIF-1α target gene transcription.

synthesis, which is regulated in an oxygen independent manner, and its degradation, which is regulated in an oxygen dependent manner[9].

In the presence of oxygen or "normoxia," prolyl hydroxylases modify proline residues 402 and 564 in the NH_2-terminal transactivation domain of the oxygen-dependent degradation domain of HIF-1α, which allows for the interaction of this subunit with von Hippel Lindau (VHL) tumor suppressor protein[10-12]. VHL is then recognized by E3 ubiquitin-protein ligase which targets HIF-1α for ubiquitination and proteasomal degradation[13,14]. In addition to hydroxylation of proline residues, hydroxylation of the asparagine 803 residue in the carboxy-terminal transactivation domain (C-TAD) of HIF-1α (in the setting of normoxia) *via* factor inhibiting HIF-1 (FIH-1) blocks transcriptional coactivation of HIF-1α with p300 and CREB binding protein (CBP), and hence inhibits transcription of target genes[15,16] (Figure 2).

HIF-1α and hypoxia

Under hypoxic cellular conditions, hydroxylation decreases due to inactivation of proline hydroxylases, leading to the inability of VHL to bind to HIF-1α and diminishes the degradation of HIF-1α. Stabilized HIF-1α, in turn, accumulates and translocates from the cytoplasm into the nucleus, where it dimerizes with HIF-1β and interacts with cofactors, such as p300/CBP, to bind to DNA on HREs, ultimately activating target gene transcription and mRNA, and eventually protein synthesis (Figure 2).

HIF-1α synthesis

In addition to this oxygen dependent mechanism of regulation leading to degradation, HIF-1α synthesis is mediated by growth factor binding to tyrosine kinase receptors, causing an activation of the phosphatidylinositol 3-kinase (PI3K) and ERK mitogen-activated protein kinase (MAPK) pathways, which represent the primary pathways responsible for cell proliferation and survival[17]. PI3K activates Akt and mammalian target of rapamycin (mTOR). In the MAPK pathway, a series of kinase activation occurs from Ras ultimately to ERK. Both the PI3K and MAPK pathways converge in activating proteins that upregulate the translation of HIF-1α mRNA into protein (Figure 3).

HIF-1α: Function in cancer

Nearly 100 HIF-1 target genes have been identified[18,19]. Transcription of these target genes produce factors essential for tumorigenesis, such as angiogenesis, glucose metabolism, survival, invasion and metastasis[18,20]. Directly activated by HIF-1, VEGF is a potent growth factor stimulating proliferation of endothelial cells and promoting angiogenesis, particularly in areas of hypoxia[21]. Furthermore, hypoxia and HIF-1α cause an increased production in enzymes and glucose transporters involved primarily in oxygen-independent, anaerobic glycolysis[22,23]. Hypoxia and HIF-1α induce growth factors, such as insulin-like growth factor-2 and transforming growth factor-α, which bind to their receptors, inducing a signal transduction cascade leading to cell proliferation and survival, and in turn stimulating further production of HIF-1α[19]. To promote invasion and metastasis, HIF-1α induces a process called epithelial-mesenchymal transition by suppressing E-cadherin, which plays a role in maintaining epithelial integrity[24,25]. The reduction of E-cadherin therefore will leave more space for tumor cells to invade through the epithelial layer and eventually metastasize. In addition, HIF-1α upregulates expression of matrix metalloproteinases, which have been associated

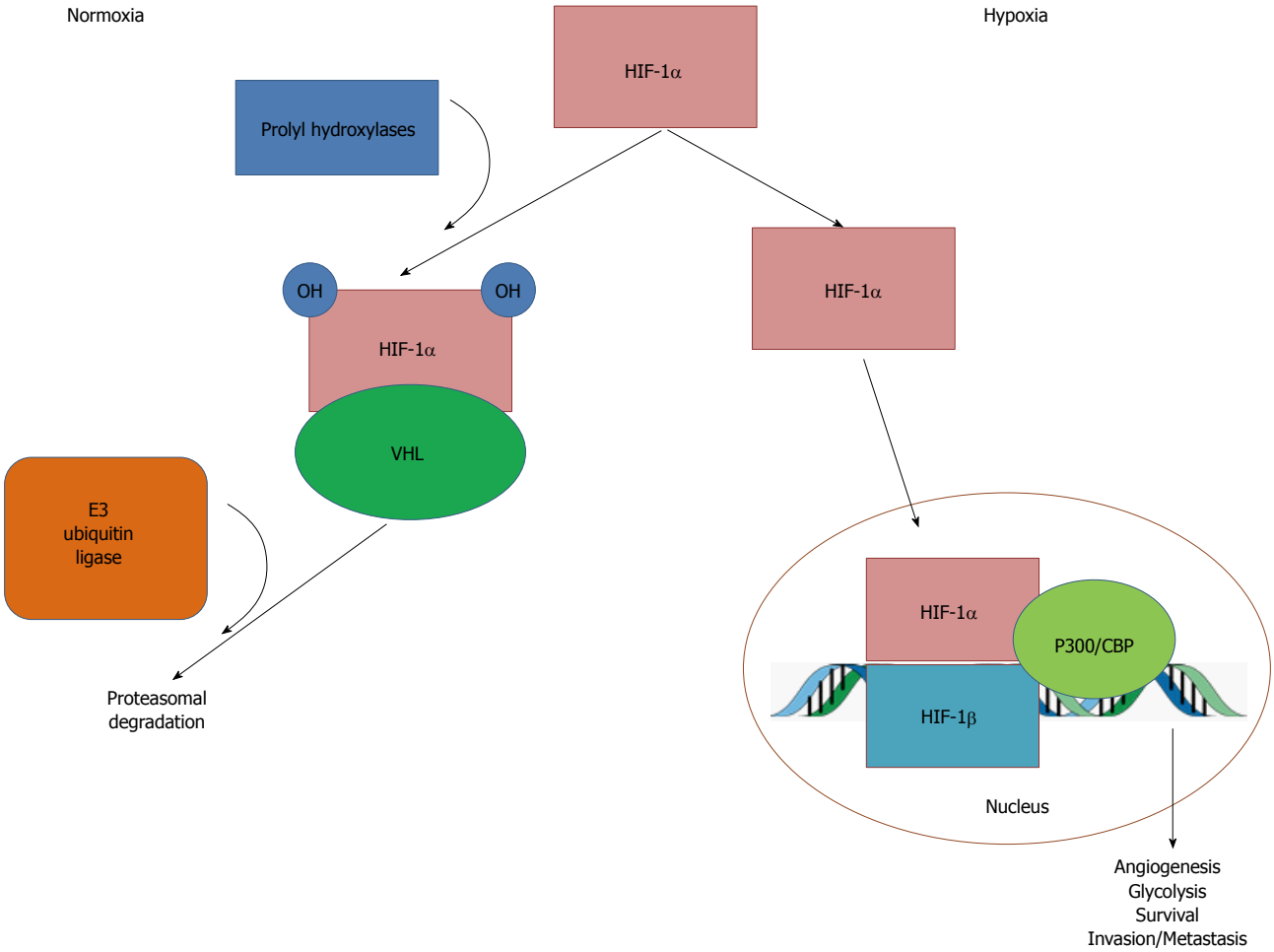

Figure 2 Oxygen-dependent mechanism of hypoxia-inducible factor-1α degradation. In the presence of oxygen (normoxia), hypoxia-inducible factor (HIF)-1α undergoes hydroxylation *via* prolyl hydroxylases. This causes HIF-1α to interact with von Hippel Lindau (VHL) tumor suppressor protein, which is in turn recognized by E3 ubiquitin ligase, which targets HIF-1α for ubiquitination and degradation. Under hypoxic conditions, reduced oxygen leads to inactivation of prolyl hydoxylases, which diminishes hydroxylation and, therefore, reduces degradation of HIF-1α. Stabilized HIF-1α accumulates and translocates into the nucleus, where it dimerizes with HIF-1β and interacts with cofactors, such as p300 and CREB binding protein, to bind to DNA on hypoxia response elements (HREs). This activates transcription of HIF-1α target genes, leading to angiogenesis, glycolysis, survival, and invasion and metastasis of cancer cells.

with the degradation of extracellular matrix (ECM) including basement membrane, removing another defense mechanism to allow tumor cells to successfully invade[26,27].

Opposite to tumorigenesis, HIF-1α also interacts with the tumor suppressor p53 gene, which in turn promotes transcription of pro-apoptotic genes. More specifically, p53 activates transcription of BAX which acts at the mitochondrial level to promote release of cytochrome C, activating a series of caspase signaling, and ultimately leading to apoptosis[28]. P53 has also been demonstrated to downregulate BCL2, an anti-apoptotic protein[29] (Figure 3). Interestingly, different downstream effects of the cell-death pathway have been reported with HIF-1α, depending on its interaction with different target genes in various types of cancer cells. In ovarian cancer cell lines, HIF-1α has been associated with better survival outcomes[30], which may be explained by the usual function of HIF-1α activating transcription of p53. However, overexpression of HIF-1α in the setting of an inactivating mutation of p53 in ovarian cancer cells has been shown to decrease apoptosis, and is associated with shorter survival in these patients[30]. In early stage esophageal cancer patients, overexpression of HIF-1α and BLC2 is associated with resistance to photodynamic therapy[31]. Furthermore, the mutation rate of p53 has been reported from 30% to as high as 67% in HCC, depending upon geographic region, with sub-Saharan Africa and East Asia with higher reported incidence[32,33]. HIF-1α expression in such tumors combined with a P53 mutation may potentially contribute to a worse prognosis in HCC.

RELEVANCE IN HCC

Hypoxia in HCC

Hypoxia plays a significant role in HCC development. Since HCC typically arises in the setting of cirrhosis induced by chronic liver injury, fibrinogenesis that results from liver injury and cirrhosis leads to reduction in vascularization, which contributes to hypoxia[34]. As

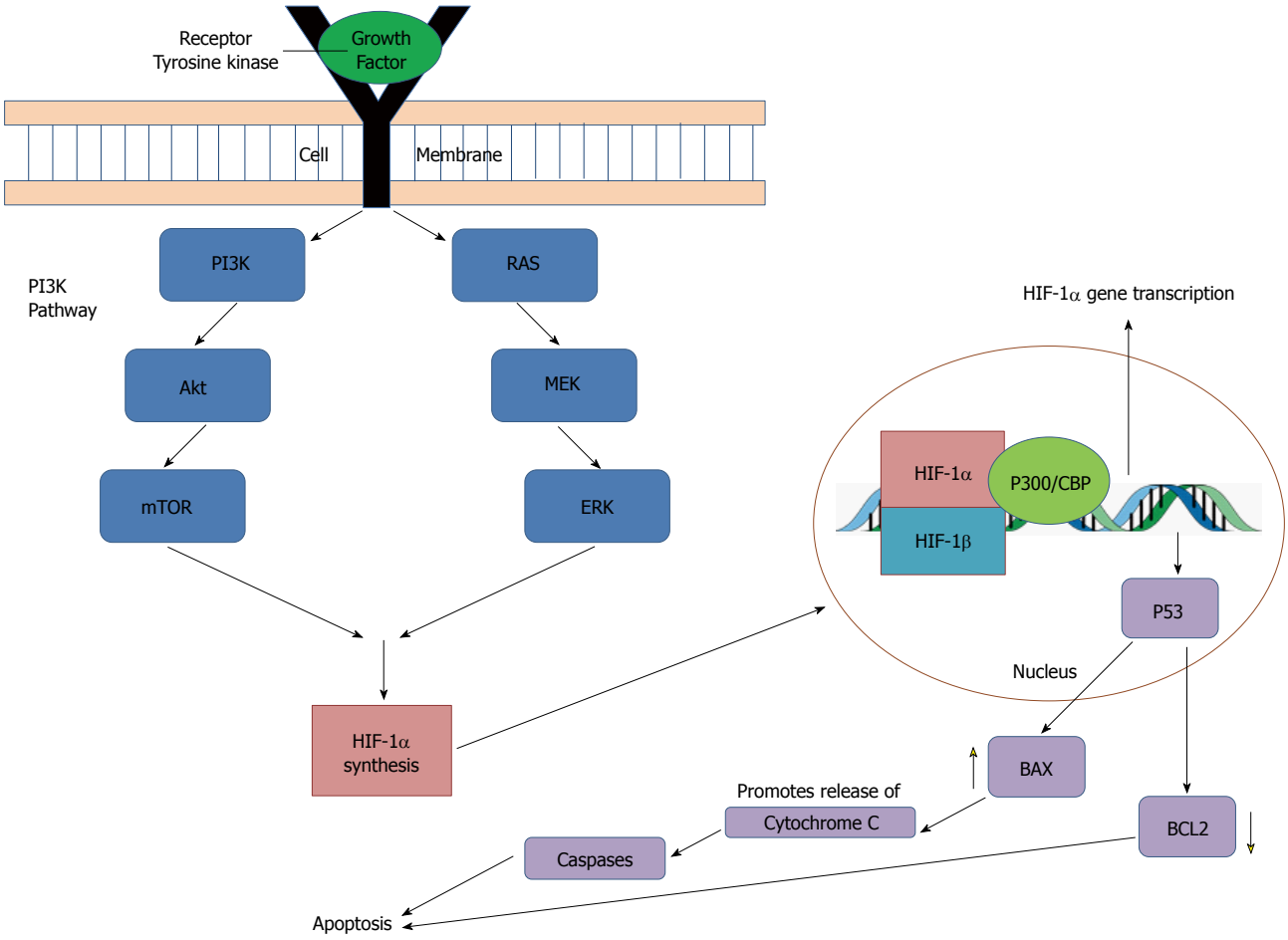

Figure 3 Hypoxia inducible factor 1α: protein synthesis and relation to apoptotis. Hypoxia-inducible factor (HIF)-1α synthesis is upregulated by growth factor binding to tyrosine kinase receptors, causing activation of two pathways essential for cell proliferation and survival: the phosphatidylinositol 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway. Extracellular signal-related kinase (ERK) and mitogen/extracellular signal-related kinase (MEK) represent members of the MAPK family which are activated as part of a signaling cascade. HIF-1α also interacts with p53, a tumor suppressor gene, which leads to transcription of pro-apoptotic genes. p53 activates transcription of BAX which acts on mitochondria to promote release of cytochrome C, activating a series of caspase signaling, which ultimately promotes apoptosis. In addition, p53 also downregulates BCL2, an anti-apoptotic protein. Together, these actions serve to increase apoptosis. mTOR: Mammalian target of rapamycin.

a result, HCC may present as cavitary lesions in the liver due to rapid growth of tumor, leading to necrosis and hypoxia. Although hypoxia can suppress cell proliferation and survival in normal cells, HCC cell lines exhibit normal cell cycle despite hypoxia, due to HIF-1α upregulated growth factors, such as VEGF which promotes tumor proliferation, and hexokinases which help generate ATP to provide an energy source for HCC cells[35,36].

HIF-1α overexpression in HCC

Identified as a poor prognostic factor in patients with varying malignancies[37], high HIF-1α expression, as measured by immunohistochemical analysis using monoclonal antibodies, has been correlated with worse clinical outcomes in patients with HCC[38]. In a study by Yang *et al*[39], HIF-1α exhibited high expression in intratumoral tumor tissue, and was closely associated with capsular infiltration and portal vein invasion; more importantly, it was associated with shorter disease free survival (DFS) and overall survival (OS). Xiang *et al*[40] evaluated HIF-1α expression in HCC patient tumor samples and correlated expression with response to treatment of abdominal lymph node metastases with external beam radiotherapy (EBRT). They found that high intratumoral HIF-1α expression was associated with worse OS rates, and lower response to EBRT. Due to its association with clinical outcomes, HIF-1α may therefore also serve as a potential biomarker for response to treatment in HCC.

HIF-1α: Pre-clinical data in HCC

Experiments in hepatoma cells by Xia *et al*[41] suggest that an interaction between HIF-1α and TNF-α leads to binding to a proliferation-specific transcription factor, Forkhead box M1 (FoxM1), enhancing proliferation of hepatoma cells and resistance to apoptosis. Furthermore, Xu *et al*[42] found that HIF-1α induced cell proliferation and cell cycle progression in hepatoma HepG2 cells by influencing expression of Cyclin A and Cyclin D. In the setting of hypoxia, HIF-1α has also been demonstrated to facilitate transcription of MDR

(multi-drug resistance) related genes in HepG2 cell lines, contributing to resistance to chemotherapeutic agents, such as 5-Fluorouracil (5-FU)[43]. The activity and relationship of HIF-1α in tumorigenesis have been examined in xenograft assays, where tumor cells are subcutaneously injected into immunodeficient mice to evaluate for gene involvement in tumor growth. Embryonic stem (ES) cells in HIF-1α knockout mice exhibit impaired vascularization in xenografts, and experience early in utero demise[44]. Although these studies involving cancer cell lines or xenografts are limited by the lack of genetic heterogeneity that is found in actual human tumors and the interactions that may occur between tumor and stromal environment, they serve as a step towards further investigations *in vivo*.

FUTURE DIRECTION: POTENTIAL THERAPEUTIC ROLE OF HIF-1α

Strategies targeting HIF-1 levels have a therapeutic potential in HCC, since they may interrupt multiple pathways implicated in angiogenesis, tumor metabolism, invasion and survival. Down-regulation of the HIF-1 complex *via* activation of hydroxylases, through inhibition of HIF-1α binding to coactivators, and through small molecule inhibitors has been studied. In a study by Knowles *et al*[45], ascorbate was found to suppress HIF-1α protein expression in human cancer cell lines, by activating hydroxylases and promoting HIF-1 degradation. Kung *et al*[46] reported reduced growth of tumor in xenograft assays *via* injection into nude mice of a fusion protein compromising GAL4 fused to the C-TAD of HIF-1α, which blocks binding of HIF-1α to its coactivators, p300/CREB. Furthermore, small molecule inhibitors, such as topotecan, a topoisomerase inhibitor, have been reported to negatively affect ribosome entry on HIF-1α mRNA, preventing translation of protein[47].

Another avenue of investigation has been the development of a HIF-1α mRNA antagonist. SPC2968 is a HIF-1α mRNA antagonist, which is a locked nucleic acid (LNA) antisense oligonucleotide, causing a down-modulation of HIF-1α mRNA and protein. LNA oligonucleotides represent a new class of nucleic acid analogs, in which conformational changes in the chemical structure lead to higher affinity for mRNA and higher potency in downregulation. This agent has undergone phase I testing in advanced malignancies to find the maximum tolerated dose and dose limiting toxicities. Though a reduction in tumor size was noted, there was no correlation with clinical efficacy. A further exploration of this HIF-1α mRNA antagonist (RO7070179) is underway in a Phase Ib proof-of-mechanism trial investigating this agent in patients with HCC after failure of at least one line of systemic therapy[48].

The therapeutic potential for HIF-1α directed therapy also lies in the possibility of combining treatment with other targeted therapies to enhance efficacy and prevent resistance. For example, a HIF-1α inhibitor may be combined with drugs that target the MAPK-RAF-ERK pathway, such as Sorafenib and Regorafenib. Liang *et al*[49] reported the ability to overcome intratumoral hypoxia-related Sorafenib resistance in HCC cells by treating them with EF24, which causes VHL-dependent HIF-1α degradation and NF-κB inactivation. Combination of HIF-1 inhibitors with mTOR inhibitors, which act upstream of HIF-1α, such as Everolimus, may also downregulate synthesis of HIF-1α and attenuate downstream signaling. Given the regulatory role of HIF-1α in apoptotic pathways, combination therapy with Stat3 inhibitors, which upregulate expression of p53 (downstream of HIF-1α, or with BCL2 inhibitors (also downstream of p53), would increase cancer cell apoptosis and augment the effect of HIF-1α inhibition (Figure 2).

Though HCC is considered relatively chemoresistant, possibly owing to a HIF-1 induced increase in multidrug resistance gene expression[50], a HIF-1α inhibitor may ultimately aid in absorption of systemic chemotherapy agents, such as doxorubicin, and enhance its effectiveness in HCC[51].

Finally, while early phase studies demonstrated no clinical efficacy with HIF-1α mRNA antagonists, the finding of tumor size reduction with therapy may be clinically significant for HCC patients who are not transplant candidates due to large tumor size. Given that there is no effective treatment which significantly reduces tumor size, a therapeutic intervention to downstage tumor may open the possibility of liver transplant in these patients for whom cure was previously not considered.

Understanding the biology and various pathways implicated in the pathogenesis of HCC is essential for the development of effective targeted interventions in advanced HCC. HIF-1α inhibition, particularly in combination with other therapies, is a promising area of research with the potential to help further the advances in systemic treatment of HCC.

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Autoimmune gastritis: Pathologist's viewpoint

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Abstract

Western countries are seeing a constant decline in the incidence of *Helicobacter pylori*-associated gastritis, coupled with a rising epidemiological and clinical impact of autoimmune gastritis. This latter gastropathy is due to autoimmune aggression targeting parietal cells through a complex interaction of auto-antibodies against the parietal cell proton pump and intrinsic factor, and sensitized T cells. Given the specific target of this aggression, autoimmune gastritis is typically restricted to the gastric corpus-fundus mucosa. In advanced cases, the oxyntic epithelia are replaced by atrophic (and metaplastic) mucosa, creating the phenotypic background in which both gastric neuroendocrine tumors and (intestinal-type) adenocarcinomas may develop. Despite improvements in our understanding of the phenotypic changes or cascades occurring in this autoimmune setting, no reliable biomarkers are available for identifying patients at higher risk of developing a gastric neoplasm. The standardization of autoimmune gastritis histology reports and classifications in diagnostic practice is a prerequisite for implementing definitive secondary prevention strategies based on multidisciplinary diagnostic approaches integrating

endoscopy, serology, histology and molecular profiling.

Key words: Autoimmune gastritis; Metaplasia; Carcinoids; Operative link for gastritis assessment staging

Core tip: Autoimmune gastritis (AIG) is an emerging gastropathy with a significant epidemiological and clinical impact on Western populations. Despite a better understanding of the phenotypic changes or cascades occurring in this autoimmune setting, the etiopathogenic mechanisms behind the disease are still poorly understood, histology reporting is not standardized, and both the AIG-associated cancer risk and its secondary prevention strategies remain confusing.

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INTRODUCTION

The clinical relationship between gastric inflammatory diseases and pernicious anemia was foreseen early in the last century. In 1935, in "*Gastritis and its consequences*", Knud Faber wrote: "…many observations agree with the hypothesis of gastritis as the cause of the anacidity, which is generally found in patients with pernicious anaemia. All these problems that are associated with pernicious anaemia, and the other forms of anaemia we find in patients with anacidity, must for the moment be approached with a certain going. …It is not improbable that similar conditions as in pernicious anaemia are to be regarded as the cause of simple microcytic anacid anaemia…. In 1913, I mentioned a typical case of this disease (*i.e.*, pernicious anemia) where the microscopical examination post mortem showed a diffuse follicular gastritis"[1]. In 1935, all that was known about the bacterium now called *Helicobacter pylori* (*H. pylori*) came from Bizzozero's microscopic observations in the stomach of dogs[2], but it is safe to assume that most, if not all of Faber's patients had *H. pylori*-associated gastritis.

In Western populations the declining incidence of infectious diseases coincides with an increasing prevalence of autoimmune conditions[3-5]. This situation also applies to the gastric setting, where the declining incidence/prevalence of *H. pylori* infection (in the West, at least) parallels a rising clinical impact of autoimmune gastritis (AIG)[6].

While the natural history of AIG is generally well defined, several crucial aspects of this condition remain unclear: the pathogenesis of autoimmune aggression is poorly understood, the clinical course of the disease in individual patients is difficult to predict, and no specific therapy is available[7].

Regarding the pathogenesis of the autoimmune response, two - not necessarily exclusive - models have been proposed. In one, considered most likely to be seen in areas where the prevalence of *H. pylori* infection is high, immune responses mounted against *H. pylori* antigens start cross-reacting with antigens that may be within the proton pump proteins or produced by the host's gastric mucosa (the intrinsic factor). This leads to a cascade of cellular responses that damage and eventually destroy the oxyntic mucosa, which stops producing acid and thus becomes both functionally and morphologically atrophic. In the second model these same events would occur irrespective of any presence of *H. pylori* infection, as a "primary" or "pure" autoimmune disorder[8].

From the clinical point of view, in addition to an impaired absorption of vitamin B_{12}, which eventually results in the clinical picture of pernicious anemia, AIG has been associated with an increased risk of two different neoplastic diseases: gastric neuroendocrine tumors (NETs; previously referred as gastric carcinoids), and adenocarcinoma[9].

This review focuses on how and when pathologists can contribute to a multidisciplinary approach to the diagnosis and clinical management of patients with AIG.

EPIDEMIOLOGY

Several studies suggest that AIG is underdiagnosed. Pernicious anemia - the most readily recognizable clinical sign of AIG - is only seen in advanced disease, and microcytic anemia (possibly an earlier sign of gastric autoimmunity) is frequently treated without thoroughly investigating its underlying cause[10].

AIG is more common in women and older people. In the general population, its prevalence has been estimated to vary between 2% and 5%, but - for the reasons outlined above - these estimates may be largely biased by the epidemiological context, comorbidities and patient selection[10]. AIG has traditionally been associated with Northern European descent, but recent studies do not support such a racial clustering. In the US, for instance, a higher prevalence of pernicious anemia has been observed among African American and Hispanic women, and an earlier age of onset has been reported in these ethnic groups too[6]. In short, the available data do not provide solid information on the incidence/prevalence of AIG in the general populations of different countries[11].

CLINICAL PRESENTATION

The clinical presentation of AIG is not associated with any specific gastrointestinal signs or symptoms[12]. In

a recent study, Miceli *et al*[13] examined the clinical and pathological features leading to a diagnosis of AIG in 99 patients: the most common initial findings were hematological disorders (various forms of anemia accounted for 37% of cases), followed by a histology positive for gastritis (34%). In less than 10% of the patients, a clinical suspicion of AIG was aroused by the concomitant presence of other autoimmune diseases, celiac disease, neurological symptoms, or a positive family history.

Microcytic anemia, the expression of iron deficiency, is a common presenting sign and is caused by achlorhydria, which impairs iron absorption[13]. In a study by Hershko *et al*[14], up to 30% of patients with iron-deficiency anemia and no clinical evidence of blood loss were found to have AIG. Advanced disease is clinically more obvious, when progressive parietal cell loss results in severe cobalamin deficiency and ultimately leads to macrocytic-megaloblastic anemia coexisting with neurological symptoms and atrophic glossitis[15].

The association between AIG and other autoimmune diseases is well recognized. Several reports have highlighted a significant association with type Ⅰ diabetes mellitus[16]. AIG may also coexist with polyglandular autoimmune (PGA) syndromes. Pernicious anemia occurs in 10% to 15% of patients with PGA type 1 syndrome (hypo-parathyroidism, Addison's disease, diabetes mellitus, and mucocutaneous candidiasis), and in 15% of PGA type 3 patients (with diabetes mellitus, and autoimmune thyroid diseases)[17,18]. The most common association, however, is with autoimmune thyroiditis ("thyrogastric autoimmunity"): more than 50% of AIG patients have circulating anti-thyroperoxidase antibodies[10]. Significant associations have also been reported with vitiligo, alopecia, celiac disease, myasthenia gravis, and autoimmune hepatitis[17].

PATHOGENESIS

Autoimmune gastritis is the result of a complex interaction between host-related factors (genetic susceptibility) and environmental factors (both endogenous and exogenous: the so-called "exposome"[19]). The resulting immunological dysregulations involve sensitized T lymphocytes[20] and autoantibodies against the parietal cell proton pump, and intrinsic factor.

The molecular grounds for the pathogenesis of AIG, and particularly the initial events that precipitate the autoimmune response, have yet to be fully elucidated. The tissue damage results from an antibody-mediated destruction of the parietal cells due to a selective targeting of the H^+/K^+ ATPase proton pump. This non-self-limiting process also induces a progressive loss of zymogenic chief cells, possibly mediated by sensitized lymphocytes[21]. Eventually, the vanishing oxyntic mucosa cells are replaced by mucous cells and metaplastic glands (of both intestinal and pseudopyloric type). In the advanced stages of the disease, the mucosa of the gastric corpus is completely replaced by atrophic and metaplastic epithelium, with no oxyntic glands remaining, so acid production may be completely lacking.

Several studies have addressed the role of *H. pylori* infection in AIG pathogenesis, and there is solid evidence to support a mechanism of molecular *mimicry* between *H. pylori* antigens and the proton pump[22]. Epidemiological studies suggest that a significant number of AIG patients have had, or still have *H. pylori* infection, and anti-proton pump autoantibodies have consistently been demonstrated in *H. pylori*-infected patients. Healthy individuals may have H^+/K^+ ATPase-autoreactive $CD4^+$ve T cells that have escaped negative thymic selection. Given a particular HLA background (*i.e.*, HLA-DR2/HLA-DR4 or HLADR4/HLADR5), *H. pylori* infection may abrogate the $CD4^+$ve $CD25^+$ve regulatory T cells that usually suppress the activation of autoantigen-presenting dendritic cells[23,24].

The Japanese scientific literature strongly supports the theory that AIG never occurs in the absence of a triggering *H. pylori* infection[25] (Sato, personal communication), but this conviction is at odds with the well-established evidence of AIG occurring in *H. pylori*-naïve patients too[8]. A study by Zhang *et al*[26] on 9684 patients (53% *H. pylori*-positive and 55% female) documented that an age-dependent prevalence of anti-parietal cell autoantibodies (APCAs) was only detectable among *H. pylori*-negative subjects. More importantly, the association observed between APCAs and atrophic gastritis was stronger among *H. pylori*-negative (OR = 11.3; 95%CI: 7-17) than among *H. pylori*-positive patients (OR = 2.6; 95%CI: 2-3).

DIAGNOSIS

Serological biopsy

The clinical value of combinations of serological tests for assessing the morphological and functional status of the gastric mucosa has been extensively addressed[6]. In AIG, the typical serological profile includes autoantibodies against intrinsic factor and parietal cells. APCA levels and hypergastrinemia correlate significantly with the inflammatory involvement of the oxyntic mucosa and serum levels of anti-intrinsic factor autoantibodies correlate with mucosal atrophy[21].

Loss of parietal cells results in significant functional changes. The higher gastric pH leads to both antral G cell hyperplasia and hypergastrinemia. The loss of chief and mucous neck cells from the oxyntic glands induces a progressive decrease in serum Pg Ⅰ, while the levels of Pg Ⅱ (sustained by the normal secretion of the unaffected antral glands) do not change significantly. This situation leads to a declining Pg Ⅰ/Pg Ⅱ ratio, which gives rise to the typical serological profile of gastric autoimmune disease (hypergastrinemia and gradually lower Pg Ⅰ/Pg Ⅱ ratios)[18-20]. Several studies

have reported on the diagnostic usefulness of the Gastropanel test (Biohit Oy, Finland) in the assessment of AIG patients[27-29].

Histology

The gastric mucosa gradually reveals a spectrum of lesions ranging from minimal inflammatory disease to severe corpus-restricted atrophic gastritis[30]. Inflammation restricted to the oxyntic mucosa is generally considered the most reliable phenotypic marker of AIG, but such an unequivocal topographical picture is rare, particularly when concurrent *H. pylori* infection induces antral gastritis.

Long-term follow-up studies suggest that early AIG exhibits no distinctive gross changes. In the absence of *H. pylori* infection, the antral mucosa is essentially devoid of inflammation and the epithelium is either normal or shows foveolar hyperplasia ("reactive gastropathy"), probably related to the trophic effects of hypergastrinemia[31]. As the oxyntic atrophy progresses, the corpus and fundus mucosa becomes irregularly flattened. The uneven loss of oxyntic glands gives rise to a grossly pseudo-polypoid appearance, with lesions consisting of foci of spared oxyntic native mucosa amidst the atrophic areas. This macroscopic appearance emphasizes the need to obtain biopsies from both non-polypoid and polypoid mucosa during endoscopic sampling procedures.

Without wishing to oversimplify the matter, it may be useful for explanatory purposes to separate the distinctive histological features of the oxyntic mucosa in AIG into four basic, sometimes overlapping phases.

Firstly, the earliest phase features an uneven infiltration of plasma cells and lymphocytes involving the full thickness of the lamina propria, with a mainly top-down gradient. The lymphocytes may be organized in nodular or follicular structures. Eosinophils and rare neutrophils can occasionally be seen. Mononuclear cells may be found intimately connected with glandular epithelial cells (intra-epithelial mononuclear infiltrate). There may be a patchy destruction of oxyntic glands.

Secondly, in a later phase of the disease, lymphocytes and plasma cells form a dense infiltrate in the lamina propria, while atrophic glandular alterations become clearly visible. In the oxyntic tubules, the native population of parietal and chief cells alternates with a new phenotype of clear, mucosecreting epithelia, with the phenotype of antral glands ("oxyntic antralization"). This metaplastic process is known as pseudo-pyloric metaplasia, or (as more recently proposed) as spasmolytic polypeptide-expressing metaplasia (SPEM). The etiological specificity of these metaplastic cells is questioned. It has been suggested that they may be pathogenically linked to any longstanding oxyntic inflammation; and this interpretation is consistent with their occurrence in inflammatory gastric polyps and Crohn-associated gastritis. On the other hand, recent experimental studies suggest that SPEM may also result from toxic damage to parietal cells (caused, for example, by DMP-777, an elastase inhibitor that ablates parietal cells without eliciting an inflammatory response)[32]. It has been demonstrated that parietal cell disruption also results in the dysregulation of parietal cell-derived signaling molecules involved in the proper differentiation of zymogen-secreting chief cells[33]. Whatever its pathogenic pathway, this SPEM population is believed to derive either from the transdifferentiation of mature chief cells[34], or from progenitor cells in the neck of the oxyntic glands[32]. SPEM cells are characterized by newly-expressed immunohistochemical markers, such as HE4 and Tff2, neither of which are expressed in native oxyntic glands. On the other hand, the original oxyntic commitment of pseudopyloric metaplastic epithelia may be disclosed by their chimeric expression of Pg Ⅰ, as documented by immunohistochemistry.

Thirdly, gastric gland intestinalization may develop in native oxyntic glands, or it may follow after an earlier pseudopyloric transformation. This last hypothesis relies on the immunohistochemical neo-expression of HE4 in both SPEM and intestinalized glands[35,36]. According to Goldenring[36], SPEM progression to intestinal metaplasia probably gives rise to a hyperproliferative state prone to genetic instability, potentially leading to gastric malignancy.

Lastly, advanced AIG is characterized by a marked reduction or complete absence of oxyntic glandular units (oxyntic mucosa atrophy), which are replaced by fibrosis of the lamina propria ("oxyntic mucosa desertification"), and pseudo-pyloric and intestinalized metaplastic glands. Other common features include hyperplasia of the muscularis *mucosae*, oxyntic pseudo-polyps, hyperplastic or inflammatory polyps, and enterochromaffin-like (ECL) cell hyperplasia. As the target of the autoimmune reaction gradually disappears, the inflammatory component becomes less prominent.

As expected, in cases of concurrent *H. pylori* infection, the antral mucosa may feature the classic spectrum of *H. pylori*-related lesions. In such cases, AIG can only be diagnosed on the strength of its specific serological profile (anti-parietal cell and anti-intrinsic factor autoantibodies)[6].

THE ELEMENTARY LESIONS OF AIG

Mucosal atrophy

Gastric mucosal atrophy, defined as the loss of "appropriate" glands, may occur in two different, usually coexisting forms. In one, the disappearing glandular units are replaced by fibrotic expansion of the *lamina propria,* resulting in a reduced glandular mass with no change in the native glandular phenotype. In the other form, native glands are replaced by metaplastic glands featuring a new commitment[37,38]. Metaplastic atrophy does not necessarily imply a

numerical reduction in the number of the glandular units, but the metaplastic replacement of native glands ultimately results in a declining population of glandular structures "appropriate" to the compartment concerned. The progressive replacement of functionally-specialized native glands with non-functioning fibrotic tissue or metaplastic cells results in the loss of the specialized functions of the oxyntic mucosa, the most evident of which is acid production.

Within the oxyntic mucosa, there may be both pseudo-pyloric metaplasia and intestinal metaplasia (IM). A third variant, pancreatic acinic cell metaplasia, may also occur. Its detection in the oxyntic mucosa of gastritis patients "should inject a strong suspicion for an autoimmune pathogenesis", but its clinical impact seems to be negligible[38].

IM is defined as the replacement of glandular or foveolar epithelium by intestinal-type epithelium. In AIG, IM arises in previously-antralized oxyntic glands (*i.e.*, from pseudo-pyloric metaplasia). Based on its histological features on hematoxylin and eosin staining, IM has been divided into two main subtypes: small-intestinal and colonic. Histochemical methods [staining mucins with high-iron diamine (HID)] help to distinguish Type Ⅰ (incomplete, or small-intestinal type) IM from Types Ⅱ and Ⅲ (complete, or colonic-type), depending on the acidity of the mucus and the morphology of the mucosecreting epithelia. Type Ⅲ features sulfomucins in the columnar epithelium and is generally regarded as the IM subtype associated with the highest risk of neoplastic transformation[39]. Intestinalization of the gastric glands parallels the induction of the intestinal transcription factor Cdx2, which results in both MUC2 upregulation, and a decreased expression of MUC5AC[40].

Neuroendocrine cell hyperplasia

The gastric hypochlorhydia or achlorhydria associated with advanced AIG stimulates sustained gastrin secretion by the antral G cells, and the resulting hypergastrinemia triggers ECL cell proliferation. ECL cells are involved in the synthesis of the molecules responsible for histamine processing (histidine decarboxylase, and vesicular monoamine transporter type 2), and possibly for histamine secretion, which in turn stimulates acid secretion from adjacent parietal cells[6,12].

In advanced AIG, ECL cells may feature a broad spectrum of changes, ranging from "true" hyperplasia to endocrine neoplasia.

The earliest lesion is linear ECL cell hyperplasia, conventionally described as a set of five adjacent chromogranin-expressing ECL cells lining the glandular neck region. ECL cell hyperplasia is also known to develop from the hypergastrinemia caused by the long-term administration of proton pump inhibitors (PPIs), although the use of PPIs has not been associated with gastric neuroendocrine tumors (NETs)[41]. The term "micronodular hyperplasia" is applied to more advanced lesions that form clusters of neuroendocrine cells (not exceeding the diameter of a gastric gland, *i.e.*, < 150 μm) surrounded by basement membrane. Five or more clusters of micronodules are defined as adenomatoid hyperplasia, a confusing term rarely used in practice[6].

ECL cell dysplasia refers to individual nodules (larger than 150 μm in diameter) with no evidence of basal membrane. Dysplastic nodules may progress to microinvasive tumors infiltrating the lamina propria, associated with perinodular fibroplasia. The factors that may contribute to neoplastic progression include mutations in the *MEN1* or *REG* genes, which provide a negative feedback for gastrin secretion[42].

It has been demonstrated in a transgenic mouse model that, under certain conditions, progenitor cells of the oxyntic glands may give rise to a cell population characterized by the loss of native parietal cells marker (*i.e.*, H/K-ATPase) expression, the neo-expression of neuroendocrine antigens (chromogranin A), and a phenotype on electron microscopy consistent with entero-endocrine cells[43]. These findings may point to a possible morphogenetic pathway for neuroendocrine tumors associated with AIG.

Neuroendocrine tumors

Micro endocrine tumors (*i.e.*, micro carcinoids) are nodules of ECL cells (ranging from 0.5-5 mm in diameter) that escape detection at endoscopy. When the diameter of these neuroendocrine proliferations exceeds 5 mm, they become endoscopically detectable and are histologically categorized as NETs.

Gastric NETs are relatively rare, with an annual incidence between 0.4% and 2%. Amongst all NETs, the US SEER database has demonstrated a rising incidence of gastric NETs: from 2.2% between 1950 and 1969 to 6.0% between 2000 and 2007. They account for 0.6%-2% of all gastric polyps identified at endoscopy[44]. The rising incidence/prevalence of gastric NETs may be due to the increasing number of endoscopy procedures performed in AIG patients, and to a greater capacity to recognize these tumors.

Three different types of gastric NET have been described: (1) NETs arising in corpus-predominant atrophic gastritis (Type 1); (2) NETs associated with gastrinoma and MEN-1 syndrome (Type 2; due to inactivating mutations in the *MEN1* and *REG* genes, which provide a negative feedback for gastrin secretion[15]); and (3) sporadic NETs (Type 3).

AIG-related NETs (*i.e.*, Type 1 NETs) account for 70% to 80% of all gastric neoplasms deriving from ECL cells. Type Ⅰ carcinoids are significantly associated with pernicious anemia: a 13-fold higher risk of AIG-related NETs emerged among 4000 Swedish patients with pernicious anemia[45].

These tumors are often multiple, small (< 1 cm in diameter), with the endoscopic appearance of a sessile

polyp in the oxyntic mucosa. Clinically, they are rather indolent and functionally "mute", and the prevalence of regional nodal metastases is less than 5%. These tumors are mostly restricted to the mucosa or submucosa and consist of well-differentiated monotonous cells, with a weak proliferative activity. Because of their peculiar phenotype/immuno-phenotype, their consistent expression of neuroendocrine markers (*i.e.*, chromogranin, synaptophysin, and CD56), and their clinical-pathological setting, gastric NETs are usually easy to diagnose. Mib1 staining is needed to identify proliferating cells and establish the tumor's grade (which can be done by using the Mib1 labeling index or the mitotic count)[46].

The overall survival rate for patients with gastric NETs is similar to that of the general population, *i.e.*, 95% at 5 years and 74% at 10 years[46].

Although the WHO recommends avoiding the historically-used term "carcinoid", which should only be applied to the clinical syndrome, well-differentiated gastric NETs are still commonly referred to as carcinoids.

Oxyntic pseudo-polyps

Against a background of atrophic AIG, the remnants of unaffected oxyntic mucosa may have the endoscopic features of pseudo-polypoid lesions. These areas are endoscopically similar to their intestinal counterparts in inflammatory bowel diseases. Histologically, they consist of oxyntic mucosa spared by the atrophic and metaplastic process, but often showing moderate chronic inflammation.

Hyperplastic polyps

Hyperplastic polyps are meta-inflammatory proliferations of the gastric foveolar cells. They are characterized by elongated, branching, and dilated hyperplastic foveolae lying in an edematous, hypervascular, inflammatory stroma. Superficial erosions are common, and result in chronic blood loss, further contributing to these patients' iron-deficiency anemia. While hyperplastic polyps arising in patients with AIG are morphologically similar to those encountered in other types of gastritis, they more often tend to be proximally located and multiple.

Pyloric gland adenoma

Pyloric gland adenomas are relatively rare tumors, usually occurring in females and AIG patients[3]. Pyloric gland adenoma prevails mainly among AIG patients (51% of a German cohort of 373 patients, and 77% of a series of 189 patients in the United States). The most common site of the lesion was the oxyntic mucosa (54%), followed by the cardia region (17%) and gastric bulb (8%)[47].

Pyloric gland adenoma consists of closely-packed pyloric-type glands, lined with cubic or columnar mucus-secreting cells that express both MUC6 and concavalin A. Although the adenomatous cell population is bland, dysplasia may occur, and there have been reports of progression to pyloric-type gastric adenocarcinoma[48-50].

Gastric adenocarcinoma

Longstanding atrophic AIG is considered a pre-cancerous condition[41]. In a Swedish cohort of 4000 patients with pernicious anemia, a 20-year follow-up documented a three-fold risk of gastric cancer (GC)[45]. The most important risk factors include clinical evidence of pernicious anemia, severity of atrophy, the presence of intestinal metaplasia, the duration of the disease, and age over 50 years[9].

In a T-cell receptor transgenic mouse model, Nguyen *et al*[51] recently demonstrated that an immunological attack against a peptide from the parietal cell antigen H/K ATPase may result in the whole spectrum of histological lesions associated with AIG. By 2-4 mo of age, these transgenic mice develop severe oxyntic atrophic/metaplastic lesions (including SPEM), higher levels of mRNA for cancer biomarkers (HE4, OLFM4, TFF2) and of phosphorylated STAT3, and high-grade intra-epithelial neoplasia.

Few studies have addressed the difference in the cancer risk between *H. pylori*-associated (*i.e.*, "secondary") AIG and "primary" AIG. This is not surprising, given the difficulty of reliably distinguishing between the two. While some studies suggested a higher cancer risk associated with corpus gastritis, they all contained significant confounding factors. For instance: (1) most of them involved patients with concomitant/previous *H. pylori* infection; (2) the atrophic/metaplastic lesions in the antrum and corpus mucosa were not scored separately; and (3) the prevalence of concomitant autoimmunity was not explored. The gastric cancer risk associated with "primary" as opposed to "secondary" AIG consequently remains unknown[52,53].

THE PATHOLOGIST'S ROLE IN ASSESSING AIG: THE DIAGNOSTIC IMPACT OF BIOPSY MAPPING

The definitive diagnosis of AIG results mainly from a combination of clinical findings (hematological profile, anti-parietal cell antibodies, serum gastrin), and histology (Figure 1). For cases with a presumptive diagnosis of AIG, the histological assessment demands a topographically defined biopsy sampling procedure that is uncommonly performed in most practices, where one or two antral specimens are often all that is taken. Pathologists are therefore rarely provided with an optimal set of samples for the purpose of even suggesting a diagnosis of AIG.

The assessment of a case relies on effective communications between gastroenterologist and pathologist. Whenever significant inflammation,

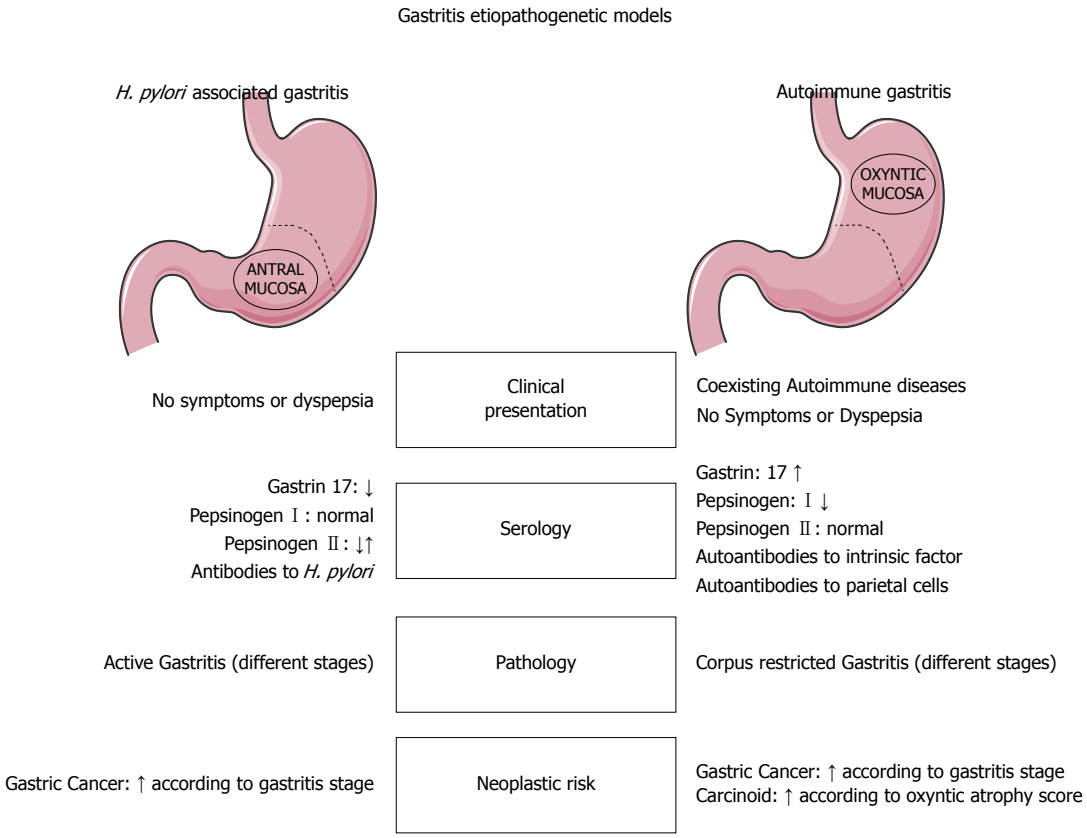

Figure 1 *Helicobacter pylori*-related *vs* autoimmune gastritis. Main differences in clinical presentation, serology (immunological and functional profiles), pathology, and neoplastic risk. *H. pylori*: *Helicobacter pylori*.

atrophy, or metaplasia are detected in the oxyntic samples in a representative gastric biopsy set, and the antral mucosa only shows minimal or reactive changes, it is incumbent on the histopathologist to report a strong suspicion of an autoimmune component, even in the absence of other clinical information to support this impression.

Early AIG is essentially impossible to diagnose and even difficult to suspect. According to the Sydney system, the diagnosis of gastritis should be based on the separate assessment of at least three samples from the antrum (including the incisura angularis), and two from the gastric body; and any focal lesions should be biopsied too[54,55]. When such topographically well-defined samples are available, the presence of chronic gastritis with minimal or no active inflammation in the corpus, and a relatively normal antral mucosa in the absence of *H. pylori* infection should elicit the suspicion of an early autoimmune phenomenon.

A histological underestimation of AIG may stem from three (possibly concurrent) technical situations: (1) inconsistencies in the number of biopsy samples available (*e.g.*, only 2 specimens, 1 of antral and the other of oxyntic mucosa); (2) inconsistent mapping of the gastric mucosa (*e.g.*, four biopsy samples, all from the antrum); and (3) inappropriate sample submission procedures (*e.g.*, biopsy samples obtained from different sites, but all submitted in the same vial). Metaplastic changes can affect the oxyntic mucosa (*i.e.*, pseudo-pyloric metaplasia), so biopsy samples obtained from the corpus and fundus may be misinterpreted as coming from a naturally mucosecreting (antral) mucosa. Hence the need to submit two biopsy samples from the oxyntic mucosa, two from the antrum and one from the angularis mucosa, all in different vials. In exceptional cases, the immunohistochemical detection of the chimeric expression of Pg Ⅰ, and the rarity/absence of G cells may nonetheless support the oxyntic origin of a biopsy sample[54,55].

A schematic representation of the main diagnostic scenarios occurring in AIG histopathology is shown in Figure 2.

THE PATHOLOGIST'S ROLE IN SECONDARY PREVENTION STRATEGIES

Both pernicious anemia and atrophic gastritis are considered precancerous conditions[56]: the real GC risk associated with AIG is still debated, and concomitant risk factors (particularly *H. pylori* infection) are believed to act as cancer co-promoters, significantly increasing the magnitude of the basic risk associated with a pure autoimmune etiology. Based on this rationale, AIG patients are eligible for GC secondary

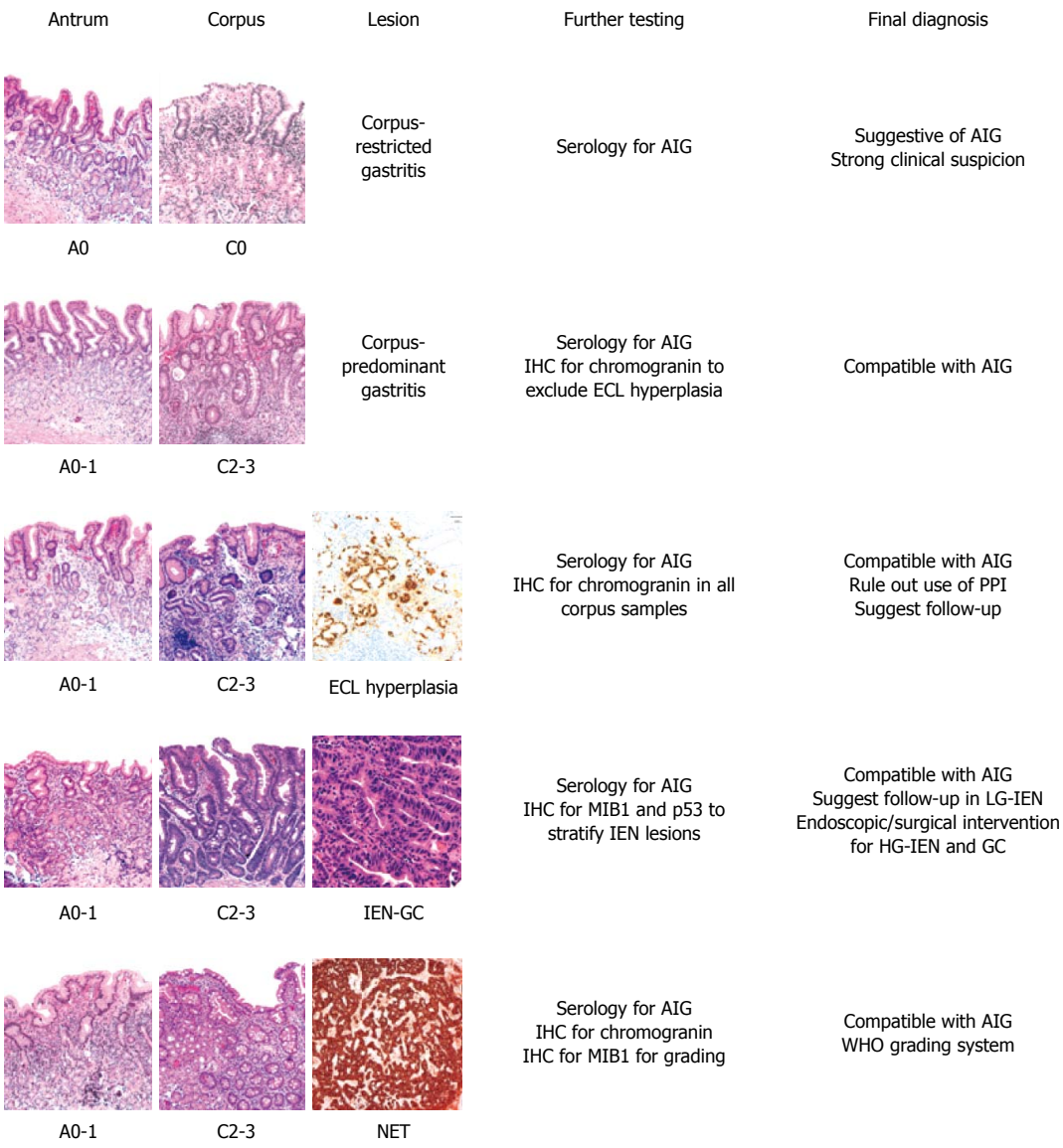

Figure 2 Schematic representation of the main diagnostic histological pictures seen in autoimmune gastritis. Metaplastic changes in the antrum and corpus are staged using the OLGA system. IHC: Immunohistochemistry; ECL: Enterochromaffin-like; IEN: Intraepithelial neoplasia; LG-IEN: Low-grade IEN; HG-IEN: High-grade IEN; GC: Gastric carcinoma; OLGA: Operative link for gastritis assessment; AIG: Autoimmune gastritis; PPI: Proton pump inhibitor.

prevention strategies. No evidence-based protocols are currently available, however, for stratifying AIG patients in different risk classes.

The American Society for Gastrointestinal Endoscopy 2006 guidelines for endoscopy of premalignant conditions recommend a single endoscopic assessment to identify neoplastic lesions, but no routine follow-up surveillance[8,57].

The European MAPS (Management of Precancerous Conditions and Lesions in the Stomach) guidelines recommend a 3-yearly endoscopic and bioptic follow-up for all patients with "extensive atrophy or intestinal metaplasia"[58]. This recommendation is based on solid evidence of the risk of cancer developing in parallel with the extent of mucosal atrophy[37]. It is important to bear in mind, however, that the greater risk associated with concomitant *H. pylori* infection has not been separately weighted.

A more objective and consistent assessment of the extent and location of atrophy has been achieved by replacing the descriptive histology report with a staging approach. The operative link for gastritis assessment (OLGA) staging system ranks gastritis in different classes of cancer risk. As in *H. pylori* infection, so too in the autoimmune setting, OLGA stages Ⅲ-Ⅳ have been significantly associated with a higher GC risk[36]. In a retrospective, single-institution study on a consecutive series of 562 AIG biopsy sets, low atrophy stages clearly prevailed (91.8%), and incidental gastric neoplasia (both intra-epithelial and invasive) only occurred in high-risk stages (Ⅲ-Ⅳ) when atrophy involved the antral mucosa as well, and was associated with *H. pylori* infection[8]. As expected, when gastritis stage was matched with endocrine cell hyperplasia/neoplasia (linear and micronodular hyperplasia and Type Ⅰ NETs) the vast majority of the hyperplastic/

neoplastic cases were in the most advanced stages (Ⅲ-Ⅳ) of gastritis[8].

Another gastritis staging system derived from OLGA, called OLGIM, relies on the histological scoring of IM alone. Because it excludes both non-metaplastic atrophy and pseudo-pyloric metaplasia from the atrophy score, the OLGIM disregards the atrophy phenotypes specifically occurring in AIG, and this results in a down-staging of AIG patients who would be considered at high risk if a global atrophy score were applied[8,59,60]. The OLGIM may therefore be less than optimal for assessing the risk of gastric cancer in AIG.

Despite the widespread availability of functional serological tests (Pg Ⅰ, Pg Ⅱ, and gastric 17, as stand-alone tests or integrated in a panel (Gastropanel, Biohit), serology is still notably absent from the international recommendations on the initial assessment and clinical monitoring of AIG. Pepsinogens and the pepsinogen ratio have been extensively validated as reliable markers of atrophy, and their low cost, accessibility, and high negative predictive value could be usefully exploited in the AIG setting. Pepsinogen Ⅰ is consistently considered a surrogate for loss of zymogenic chief cells from the oxyntic mucosa, but large studies are now available that have correlated the organic with the functional disease in AIG. Low Pg Ⅰ levels (particularly with a low Pg Ⅰ /Pg Ⅱ ratio) are consistently associated with oxyntic atrophy. Gastrin 17 is an elective marker of proton pump efficiency and is considered a reliable marker of oxyntic atrophy.

CONCLUSION

There are gaps in the information available on the epidemiology of gastric autoimmunity, and the incidence of AIG is probably underestimated. Secondary autoimmune gastric disease may be triggered by longstanding *H. pylori* infection, but "primary" autoimmune gastritis exists as a separate clinical entity as well. The clinical signs of AIG include a broad spectrum of non-hematological and hematological disorders, but megaloblastic anemia is mostly associated with advanced gastric disease.

From a clinical viewpoint, AIG can be diagnosed reliably by means of: (1) specific autoantibody assays (anti-parietal cells, and anti-intrinsic factor autoantibodies); (2) functional serology of the gastric mucosa (Pg Ⅰ/Pg Ⅱ ratio, gastrin 17); and (3) histology (applying a standard diagnostic biopsy sampling protocol).

There is clear evidence of a link between long-standing AIG and a spectrum of ECL cell changes ranging from hyperplastic to neoplastic lesions (gastric NETs).

The magnitude of the AIG-associated GC risk probably differs in "primary" *vs H. pylori*-associated, "secondary" autoimmune disease (and is presumably higher in the latter, but also in advanced "primary" AIG). Both gastric "functional serology" and histological staging are reliable in monitoring disease progression, and for stratifying patients in different prognostic classes.

No comprehensive molecular profiling is available for AIG, and no differential molecular profiling is capable of distinguishing primary from secondary autoimmune gastritis. In clinical practice, such information could make AIG assessment and prognostication more reliable, as well as supporting the rationale for biologically-targeted therapies.

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MINIREVIEWS

Research progress and prospects of markers for liver cancer stem cells

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Abstract

Liver cancer is a common malignancy and surgery is the main treatment strategy. However, the prognosis is still poor because of high frequencies of postoperative recurrence and metastasis. In recent years, cancer stem cell (CSC) theory has evolved with the concept of stem cells, and has been applied to oncological research. According to cancer stem cell theory, liver cancer can be radically cured only by eradication of liver cancer stem cells (LCSCs). This notion has lead to the isolation and identification of LCSCs, which has become a highly researched area. Analysis of LCSC markers is considered to be the primary method for identification of LCSCs. Here, we provide an overview of the current research progress and prospects of surface markers for LCSCs.

Key words: Hepatocellular carcinoma; Liver cancer stem cells; Surface markers; CD90; Epithelial cell adhesion molecule

Core tip: Liver cancer is a common malignancy and the eradication of liver cancer stem cells (LCSCs) is proposed as the key to improving the curative effect of treatments. Many surface markers have been reported for LCSCs, but there is still no unified standard. This paper addresses the research progress of markers for LCSCs and discusses the relationship with clinical

syndrome in hepatocellular carcinoma.

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DEFINITIONS OF CANCER STEM CELLS AND LIVER CANCER STEM CELLS

Liver cancer is the fifth most common and third most deadly cancer in the world and hepatocellular carcinoma (HCC) accounts for 90% of liver cancers. Currently, surgical resection and liver transplantation are the sole curative options for the treatment of HCC. However, the 5-year survival rate depends on the stage of the liver cancer at diagnosis. Most patients in the late stage with postoperative recurrence exhibit low sensitivity to radiotherapy and chemotherapy[1]. Therefore, the exploration of liver cancer treatments is currently a highly researched area. Since cancer stem cell theory was first proposed, new approaches have been suggested for the treatment of HCC. Cancer stem cell theory was put forward by Reya *et al*[2] based on the previous points. The theory considers that tumor tissue has a small number of cells called cancer stem cells (CSCs) that self-renew indefinitely and have the potential for multi-directional differentiation to produce the heterogeneity of tumor cells. Most CSCs are in the G0 phase of the cell cycle and have certain resistance to radiotherapy and chemotherapy.

Studies have demonstrated CSCs in leukemia and breast cancer. Other studies have shown that CSCs may also exist in liver cancer tissues[3,4]. Liver cancer stem cells (LCSCs) have been highly researched in liver cancer for more than 10 years, and are considered to be a population of cells with certain stem cell-like characteristics in liver cancer tissue. These stem cell-like characteristics include indefinite self-renewal and the potential for multi-directional differentiation that constantly produces liver cancer cell populations in various stages of differentiation with different biological behaviors. In this manner, LCSCs maintain tumor growth. Furthermore, compared with non-LCSCs in liver cancer tissue, LCSCs have a stronger migration ability and tumorigenicity that is closely related to metastasis and recurrence of liver cancer. LCSCs are also resistant to radiotherapy and chemotherapy, which is one of the reasons for the poor efficacy of these treatments for liver cancer patients[5,6].

An increasing number of researchers believe that the key to improving the curative effect of treatments for liver cancer is the eradication of LCSCs. Treatments of liver cancer may simply kill cancer cells and reduce the tumor volume without eradication of LCSCs. In addition, detection of LCSC-specific surface markers can be used for diagnosis, prognosis evaluation, and monitoring after treatment of patients with liver cancer.

CLASSIFICATION OF LCSC MARKERS

In recent years, numerous studies have confirmed that the development and progression of hematological cancers and many kinds of solid tumors, including liver cancer, depend on CSCs[7]. However, compared with most other solid tumors with CSC-specific surface markers, there is no consensus on the surface markers of LCSCs. New LCSC surface markers are constantly being discovered and debated.

Currently, using cell surface markers to isolate CSCs has proved to be feasible[8]. To select LCSC surface markers, stem cell markers are used as a reference because CSCs and normal stem cells have many similar biological characteristics. Major LCSC markers are listed in Table 1.

CD133

Human CD133 is a five-transmembrane single-chain glycoprotein that belongs to the prominin family, which contains two large extracellular and two small intracellular loops[9,10]. The role of CD133 as a CSC marker in liver cancer has been documented in several studies[11-13]. Suetsugu *et al*[14] reported that CD133+ Huh-7 cells (liver cancer cell line) have a high proliferative capacity *in vitro* and tumorigenic ability *in vivo*. Subsequently, Yin *et al*[15] isolated CD133+ cells from the HCC cell line SMMC -7721, and found that these cells had the highest colony-forming ability *in vitro* and tumorigenic ability *in vivo*. Yao *et al*[16] showed that knockout of the CD133 gene in Huh-7 cells suppresses their tumorigenic ability *in vivo*. Kohga *et al*[17] reported a relationship between CD133 and the invasion and distant metastasis of liver cancer. A recent study[12] showed that CD133+ liver cancer cells are resistant to apoptosis induced by radiation and have a stronger proliferative ability *in vitro* and tumorigenic ability *in vivo*. Some studies have shown that CD133+ liver cancer cells exhibit stronger abilities for colony formation and proliferation, which result in a poorer prognosis compared with CD133- liver cancer cells[18,19]. These findings indicate that CD133+ liver cancer cells may be LCSCs.

However, Salnikov *et al*[20] found that CD133+ and CD133- liver cancer cells have no significant differences in terms of migration, and the total number of CD33+ cells has no correlation with the clinical features of liver cancer patients. Therefore, CD133 as a LCSC marker requires further study.

Table 1 Reported major liver cancer stem cell markers

Marker	Origin	Minimum tumorigenic cells	Clinical relevant characteristics	Related literature
CD133	HuH7 cell, SMMC-7721 cell	1×10^2-1×10^3	With a poor prognosis; Related with invasiveness and distant metastases; Resistance to chemotherapy drugs	[14,15,17,48,59]
CD90	PLC cell, MHCC-97L, HCC tumor, blood samples of 90% patients with liver cancer	5×10^2	Related with tumorigenic ability and metastasis of liver cancer	[23,24]
EpCAM	HuH1 cell, HuH7 cell	1×10^3	The origin of recurrence and metastasis postoperatively; Patients with vascular metastasis and low overall survival rate when peripheral circulation exists	[31]
OV6	SMMC-7721 cell	5×10^3	Drugs resistance; Strong metastasis and invasion	[39]
CD44[1]	MHCC-LM3 cell, MHCC-97L cell	1×10^2	Chemotherapy drug resistance; Related with portal vein metastasis in liver cancer	[41]
SP (ABCG2)	Huh7 cell, PLC/PRF/5 cell	1×10^3	-	[46,60]

[1]Co-expression of CD133+ and CD44+ cell surface markers. SP: Side population; EpCAM: Epithelial cell adhesion molecule.

CD90

CD90 (Thy-1) is a 25-30 kDa glycosylphosphatidylinositol-anchored glycoprotein expressed on many cell types, including T cells, thymocytes, neurons, endothelial cells, and fibroblasts. It is an important regulator of cell-cell and cell-matrix interactions with important roles in nerve regeneration, metastasis, inflammation, and fibrosis[21]. A study[22] has reported that CD90 is a surface marker for liver stem cells and hepatic progenitor cells during liver development. Recently, CD90 has also received attention as a CSC marker for various types of tumor cells including hepatic stem cells.

Yang *et al*[23] reported CD45-CD90+ cells in all liver cancer tissues and 90% of blood samples from liver cancer patients. They also found high expression of CD90 during tumor formation. These findings suggest that CD90 may participate in liver cancer development. Yang *et al*[24] also found that 4×10^3 CD45-CD90+ cells from tumor tissues can form liver carcinoma in Beige/SCID mice. In addition, CD90+ cells can differentiate asymmetrically into CD90+ cells and CD90- cells, but CD90- daughter cells are all CD90- cells. These results show that CD90+ cells have strong proliferation, drug resistance and self-renewal abilities[25]. Recently, Michishita *et al*[26] reported that CD90+CD44+ HCC930599 cells (dog liver cancer cell line) have a stronger proliferation ability *in vitro* as well as self-renewal and tumorigenic abilities than CD90-CD44+ cells. All of these observations suggest that CD90 can be used as an LCSC marker.

The above studies show that CD90 is a potential marker of LCSCs. However, another study[27] reported that CD90+ liver cancer stem-like cells may participate in the late stage of liver cancer, and only appear in hepatitis B infection-related liver cancer. Therefore, CD90 still requires further research as a biomarker for LCSCs.

EPITHELIAL CELL ADHESION MOLECULE

Epithelial cell adhesion molecule (EpCAM), also known as CD326, is a single transmembrane glycoprotein encoded by the tumor-associated calcium signal transducer 1 gene, which belongs to a family of adhesion molecules. It has a molecular mass of 30-40 kDa and consists of three domains: an extracellular domain, a single transmembrane domain, and an intracellular structure domain. EpCAM has proven to be a marker of mature liver stem cells and progenitor cells, and is also a marker of hepatic oval cells[28,29]. Studies have shown that EpCAM participates in the β-catenin/Wnt signaling cascade, in which activation of proto-oncogenic proteins c-myc and cyclinA/E leads to tumorigenesis[30]. Yamashita *et al*[31] first reported that EpCAM can serve as a marker for LCSCs. Chen *et al*[32] found that CD133+EpCAM+ Huh7 cells have strong abilities for multi-directional differentiation, self-renewal, and clonal colony formation. Furthermore, only 500 CD133+EpCAM+ cells are tumorigenic in NOD/SCID mice. In addition, CD133+EpCAM+ cells show high expression of stem cell markers Nanog, Oct4, and Sox2.

In HCC patients, Sun *et al*[33] showed that EpCAM+ cells in peripheral circulation express other reported LCSC markers, CD133 and ABCG2. In NOD/SCID mice, injection of tumor cells showed that 300 EpCAM+CD45- cells were tumorigenic, whereas 1×10^4 EpCAM-CD45- cells were not tumorigenic. Schulze *et al*[34] also suggested the existence of EpCAM+ cells in peripheral circulation of patients with liver cancer.

Their clinical pathologic features tended to be > 400 ng/mL serum α-fetoprotein (AFP), various degrees of blood vessel metastasis, middle and advanced stage, and an overall low survival rate. Guo *et al*[35] followed patients with liver cancer after radical surgery, and found that the 1-, 2-, and 3-year survival rates of patients with EpCAM+ specimens were 85.7%, 51.3%, and 85.7%, respectively. Therefore, EpCAM+ cells may be LCSCs and radical surgery cannot completely kill these cells, which is the root cause of postoperative recurrence and metastasis. Therefore, EpCAM+ cell-targeting therapies are needed for the treatment of liver cancer.

OV6

Hepatic oval cells, called hepatic stem/progenitor cells in the liver Herring pipe, can differentiate into hepatocytes and bile duct cells. OV6 is a marker of hepatic oval cells[36]. In liver cancer induced by gene mutation, hepatic oval cells can become abnormal and differentiate into liver cancer cells or bile duct epithelial cells[37]. Thus, liver stem/progenitor cells may be involved in the development and progression of liver cancer. Recently, Jia *et al*[38] examined various cell surface markers, and found that liver cancer cells were derived from liver stem/progenitor cells. Yang *et al*[39] showed that OV6+ liver cancer cells have a stronger tumorigenic ability and chemotherapy resistance than OV6- cells. In addition, they found that the proportion of CD133+ cells in an HCC cell line ranged from 0.1% to 75%, while the proportion of OV6+ cells was relatively stable at 0.2%-3%. It is interesting to note the CD133+ cells express OV6, which further shows that OV6 can serve as a marker of LCSCs. Using magnetic bead separation, Yang *et al*[40] isolated OV6+ cells from HCC cell lines SMMC7721 and Huh7, and found that 103 OV6+ SMMC7721 cells or 104 OV6+ HuH7 cells were tumorigenic in NOD/SCID mice. They also found that OV6+ HCC cells *in vivo* and *in vitro* had strong invasion and metastatic abilities. These studies suggest that OV6 may be a potential marker of LCSCs.

CD44

CD44 is a transmembrane glycoprotein that mediates adhesion between cells and the extracellular matrix, lymphocyte activation and homing, and plays an important role in the invasion and metastasis of cancer. Zhu *et al*[41] reported that CD133+CD44+ cells have a strong tumorigenic ability in nude mice and high expression of the ATP binding cassette (ABC) transporter superfamily members (ABCB1, ABCC1, and ABCG2) that mediate resistance to chemotherapeutic drugs such as doxorubicin and vincristine. Further study found that CD133+CD44+ cells express genes related to stem cells such as β-catenin and Bmi-1. Hou *et al*[42] showed that CD133+CD44+ cells are the initial cells that produce metastasis to the lung and liver in immunodeficient mice. Analyses of human liver specimens showed that CD133+CD44+ liver cancer cells are associated with metastasis to the liver portal vein. Therefore, CD133 and CD44 can better define LCSCs.

Yang *et al*[23] first reported that CD90+CD44+ liver cancer cells are more aggressive, and both CD44 and CD90 can better define LCSCs. After irradiation of N1S1 rat liver cancer cells, Thompson *et al*[43] found a 22-fold increase in CD44+CD90+ cells and the use of a BEZ235 blocker could avoid thermal stress damage of PI3K-Akt-mTOR signaling pathways, in which CD44+ cells increased while CD90+ cells did not change. Further study of immunodeficient mice injected with liver cancer cells revealed CD44+ cells, but not CD90+ cells, on the edge of the thermal ablation and the edge of the liver cancer lesion. Therefore, after thermal ablation, recurrence was associated with a small number of CD44+ cells. Recently, Fernando *et al*[44] reported that transforming growth factor (TGF)-β treatment of long term-cultured PLC/PRF/5 liver cancer cells can induce resistance to sorafenib. Further analysis found that CD44 expression induced epithelial-to-mesenchymal transition characterized by vimentin protein expression, but the drug resistance was proportional to the number of CD44+ cells. In addition, repeated treatment with sorafenib could enrich CD44+ cells. Therefore, CD44 may be a potential molecular marker of LCSCs.

SIDE POPULATION CELLS

Fluorescence-activated cell sorting can isolate CSCs known as side population (SP) cells from a wide variety of tumor cell lines because the ABCG2 transporter effluxes the fluorescent DNA dye Hoechst 33342. The cell surface protein ABCG2, also called breast cancer resistance protein, is a member of the ABC transporter family, which was first identified in drug-resistant breast cancer cells. Cells expressing ABCG2 will pump out drugs, resulting in multi-drug resistance. LCSCs that are resistant to chemotherapy[45] indicate that ABCG2 is a candidate molecular marker of CSCs, which can be used for cell separation.

Chiba *et al*[46] found 0.25%-0.80% of SP cells among liver cancer cell lines Huh7 and PLC/PRF/5. Compared with non-SP cells, the SP cells had a higher proliferative capacity and considerable ability to resist apoptosis. *In vitro* studies also suggest that transplantation of 103 SP cells into immunodeficient NOD/SCID mice can cause tumors. Therefore, separation of SP cells can be useful when CSC markers are unknown. A recent study[47] reported that SP cells from the HAK-1A liver cancer cell line do not express CD90, EpCAM, CD13 or CD133. In the HAK-1B cell line, compared with non-SP cells, the SP cells have a clonal growth ability, strong tumorigenic ability, fast growth rate, and highly express CD13. However, there are no differences in the chemotherapy resistance,

colony forming ability, or cell cycle.

TARGETED THERAPIES AGAINST LCSC MARKERS

Because LCSCs are related to drug resistance, metastasis, and recurrence of liver cancer, targeting LCSCs as a cancer treatment has become a promising strategy. Some studies have found that treatment measures targeting certain surface markers of LCSCs can inhibit their self-renewal and tumorigenesis, such as disrupting the expression of LCSC surface markers CD133[48], EpCAM[31,49], CD24[50], and CD13[49]. In addition, neutralizing antibodies against CD44 can effectively induce apoptosis of CD90+CD44+ LCSCs[23]. There is a broad prospect to develop targeted drugs against specific surface markers of LCSCs.

Some key signaling pathways in LCSCs are also therapeutic targets. Single target therapy is limited, but targeting both the LCSCs and surrounding environment may be more effective to inhibit the growth and metastasis of HCC.

Determination of specific markers for LCSCs and development of corresponding diagnostic strategies will be useful to detect LCSCs and monitor whether LCSCs have diffused into the blood and bone marrow. Such approaches might accurately predict metastasis and/or recurrence in HCC patients, and enable more individualized treatment plans.

PROBLEMS AND PROSPECTS OF LCSCS

LCSC sources

CSC theory suggests that tumor growth and progression are maintained by a small population of CSCs in tumor tissue. The number of cells with stem cell properties is maintained by CSC self-renewal, while CSCs constantly produce new tumor cells by differentiation.

In terms of the origin of CSCs (including LCSCs), there are two viewpoints. Most researchers believe that CSCs originate from abnormal proliferation and differentiation of stem cells[51-53]. In chronic liver disease caused by a variety of reasons such as viral hepatitis, fatty hepatitis, and metabolic liver disease, liver stem cells are actively proliferating. Under the influence of carcinogenic factors, actively proliferating liver stem cells might undergo malignant changes. In addition, most recognized LCSC surface markers such as CD133, EpCAM, and CD90 are also surface markers of stem cells. Finally, the development of LCSCs has common molecular signaling pathways or regulatory molecules with liver stem cells, such as Wnt, TGF-β, Notch, Hedgehog, Myc, and Bmi1[6]. However, the derivation of induced pluripotent stem cells by Takahashi *et al*[54] changed the understanding of the sources of stem cells. Therefore, some researchers consider that CSCs can also be induced from mature tumor cells under the action of various factors[55,56]. Recently, Holczbauer *et al*[57] reported that liver stem/progenitor cells and mature liver cells can transform into LCSCs by excessive activation of Ras. LCSCs are a dynamic cell population. Therefore, factors in the surrounding environment (such as chemotherapeutic drugs, radiation, oxygen, growth factors, and inflammatory factors) might induce both differentiation of LCSCs to cancer cells and mature cells to LCSCs to maintain the growth and progression of tumors.

FUTURE APPLICATIONS OF LCSC MARKERS

Although many surface markers have been reported for LCSCs, there is still no unified standard. At present, cells isolated by most surface markers have LCSC characteristics, but some markers mutually have less cross expression. Therefore, each marker may represent a subset of LCSCs. For example, Yamashita *et al*[27] reported that EpCAM+ and CD90+ LCSCs represent different cells with different biological characteristics. In addition, CSCs are considered to be a small population of tumor cells, and some markers, such as CD133, EpCAM, CD44, and CD24, can be expressed by 50% or more cells in HCC cell lines. Whether these markers can fully represent LCSCs is unclear, and their sensitivity and specificity require further study. To improve the specificity of LCSC markers, some researchers have advocated the combined use of multiple markers for LCSCs, such as CD90 and CD44[23,24], CD133 and CD44[41], CD133 and ALDH[11], EpCAM and AFP[31], and CD133 and EpCAM[32]. Considering a consensus is yet to be reached for LCSC markers, these markers may represent heterogeneous cells. Which surface markers or which combination of markers has higher specificity still requires further research.

LCSC markers have not been recognized until now, and the current problem is determination of LCSC-specific markers for identification, separation and cultivation of LCSCs. Different LCSC markers may represent different stages of liver stem cell differentiation[58]. Furthermore, LCSCs of different origins may express different markers[31].

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META-ANALYSIS

Hepatocellular carcinoma in the elderly: Meta-analysis and systematic literature review

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Abstract

AIM: To conduct a meta-analysis to investigate the clinical outcomes of surgical resection and locoregional treatments for hepatocellular carcinoma (HCC) in elderly patients defined as aged 70 years or more.

METHODS: Literature documenting a comparison of clinical outcomes for elderly and non elderly patients with hepatocellular carcinoma was identified by searching PubMed, Ovid, Cochrane Library, and Web of Science databases, for those from inception to March 2015 with no limits. Dichotomous outcomes and standard meta-analysis techniques were used. Heterogeneity was tested by the Cochrane *Q* statistic. Pooled estimates were measured using the fixed or random effect model.

RESULTS: Twenty three studies were included with a total of 12482 patients. Of these patients, 6341 were treated with surgical resection, 3138 were treated with radiofrequency ablation (RFA), and 3003 were treated with transarterial chemoembolization (TACE). Of the patients who underwent surgical resection, the elderly had significantly more respiratory co-morbidities than the younger group, with both groups having a similar proportion of cardiovascular co-morbidities and diabetes. After 1 year, the elderly group had significantly increased survival rates after surgical resection compared to the younger group (OR = 0.762, 95%CI: 0.583-0.994, *P* = 0.045). However, the 3-year and 5-year survival outcomes with surgical resection between the two groups were similar (OR = 0.947, 95%CI: 0.777-1.154, *P* = 0.67 for the third year; and OR = 1.131, 95%CI: 0.895-1.430, *P* = 0.304 for the fifth year). Postoperative treatment complications were similar between the elderly and younger group. The elderly group and younger group had similar survival outcomes for the first and third year after RFA (OR = 1.5, 95%CI: 0.788-2.885, *P* = 0.217 and OR = 1.352, 95%CI: 0.940-1.944, *P* = 0.104). For the fifth year, the elderly group had significantly worse survival rates compared to the younger group after RFA (OR = 1.379, 95%CI: 1.079-1.763, *P* = 0.01). For patients who underwent TACE, the elderly group had significantly

increased survival compared to the younger group for the first and third year (OR = 0.664, 95%CI: 0.548-0.805, *P* = 0.00 and OR = 0.795, 95%CI: 0.663-0.953, *P* = 0.013). At the fifth year, there were no significant differences in overall survival between the elderly group and younger group (OR = 1.256, 95%CI: 0.806-1.957, *P* = 0.313).

CONCLUSION: The optimal management strategy for elderly patients with HCC is dependent on patient and tumor characteristics. Compared to patients less than 70, elderly patients have similar three year survival after resection and ablation and an improved three year survival after TACE. At five years, elderly patients had a lower survival after ablation but similar survival with resection and TACE as compared to younger patients. Heterogeneity of patient populations and selection bias can explain some of these findings. Overall, elderly patients have similar success, if not better, with these treatments and should be considered for all treatments after assessment of their clinical status and cancer burden.

Key words: Liver neoplasms; Hepatocellular carcinoma; Elderly patients; Cancer treatment; Meta-analysis

Core tip: The optimal management strategy for elderly patients with hepatocellular carcinoma (HCC) is dependent on patient and tumor characteristics. This meta-analysis suggests that surgical resection, transarterial chemoembolization, and radiofrequency ablation are safe and effective treatment options for elderly patients with HCC. Overall, elderly patients have similar success with these treatments compared to younger patients and should be considered for all treatments pending their clinical status and cancer burden.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, has become the fifth most common cancer and the third most common cause of cancer-related mortality worldwide[1]. The management of HCC is multi-disciplinary with a wide range of treatment options ranging from liver resection, liver transplantation, locoregional therapies including ablation and transarterial chemoembolization (TACE), and molecular-targeting therapies[1-32]. As our society continues to age, there will be an increasing proportion of elderly patients with hepatocellular carcinoma. In China and South Korea, the mean age at HCC diagnosis was found to be 55-59 years[2]. In Europe and North America, the average age of HCC diagnosis is 63 to 65 years, and up to 75 years in low risk populations[5]. Although HCC is a worldwide public health problem, there are epidemiological differences in the incidence of HCC based on geographic location. In Asia, the overwhelming etiology continues to be endemic hepatitis B. In Western countries, the most common etiologies are hepatitis C and alcoholic cirrhosis.

HCC in the elderly population has been found to demonstrate distinct clinicopathological characteristics from the disease process in the younger population. Reports indicate that elderly patients with HCC were more likely to be female, possibly due to their longer life expectancy[3]. Elderly patients with HCC were more likely to have chronic hepatitis C[3]. Most hepatitis B carriers acquire the disease in the perinatal period *via* vertical transmission, whereas hepatitis C infection is usually acquired in adulthood. As a result of earlier acquisition of the virus, the average age of diagnosis of HBV-related HCC is usually 10 years earlier than that of HCV-related HCC[1]. In the elderly population with HCC, there is also a larger proportion of patients who are negative for both hepatitis B surface antigen and HCV antibody, as compared to younger patients[4]. Elderly patients with HCC have also been shown to be more likely to have a normal liver with a lower grade of background liver fibrosis[5]. Aging is associated with decreased liver mass, hepatic blood flow, and synthesis or metabolism of exogenous substrates[5]. Whether these physiological changes are associated with worse outcomes in elderly HCC patients remains is debatable.

The effect of age on cancer treatment allocation is controversial. In general, elderly patients are thought to have increased co-morbidities including cardiovascular disease, pulmonary disease, diabetes mellitus, and renal insufficiency[1]. Whether elderly patients with HCC have equal access to and outcomes from treatment as compared to their younger counterparts is controversial, with some data to suggest increased co-morbidities and poorer functional status in elderly HCC patients[14]. In a large multicentre report by the Italian Liver Cancer group, it was shown that elderly patients with HCC were more likely to undergo percutaneous procedures and were less likely to undergo surgical resection and TACE[14]. The data regarding safety and clinical outcomes of locoregional therapies for HCC in the elderly population remains limited.

There are a wide variety of treatment options for HCC depending on the stage of disease. Current treatments include surgical resection, liver transplantation, transcatheter arterial chemoembolization, radioembolization, percutaneous radiofrequency

ablation, percutaneous ethanol injection, percutaneous microwave coagulation therapy, and molecular-targeted therapy with sorafenib. Most studies evaluating clinical outcomes of such treatments in the elderly have been limited by small sample size and have varying results[1,5,13,14]. Most reports have demonstrated comparable outcomes between elderly and younger patients[5,14,23]. Previous studies showed similar survival rates after surgical resection with the exception of Hirokawa *et al*[17] who reported better disease free survival in younger patients and Liu *et al*[20] who reported better overall survival in younger patients[18,21]. In respect to radio frequency ablation (RFA), there were two studies which showed better long term outcomes in the younger patients[26,27]. For TACE, previous studies have reported comparable outcomes with the largest study showing better outcomes in the elderly group[27]. In this meta-analysis, we analyze characteristics and clinical outcomes of elderly patients with HCC treated with different modalities including surgical resection, percutaneous radiofrequency ablation, and transarterial chemoembolizaton (TACE).

MATERIALS AND METHODS

Literature documenting a comparison of clinical outcomes in elderly patients undergoing surgical and locoregional therapies for HCC was identified by searching PubMed, EMBASE, Cochrane Library, and Web of Science databases, for those from inception to March 2015 with no limits.

The inclusion criteria for the present meta-analysis were as follows: published comparative studies reporting extractable data for survival outcomes for elderly and non elderly patients with HCC who underwent surgical resection, RFA, and TACE with elderly defined as aged 70 and above. Studies which included only Kaplan-Meier survival curves and did not include the number of annual survivals were not considered. A total of 125 references were identified through the literature search. Of these, 23 studies were included. All studies were observational and were either prospective or retrospective by design. Exclusion criteria included nonhuman studies, case reports, editorials, studies lacking control group of non elderly patients, studies in which patients were diagnosed with liver metastases or cholangiocarcinoma.

Demographics and clinical patient characteristics were thoroughly reviewed. Co-morbidities were assessed and included cardiovascular disease, respiratory disease, and diabetes. Cardiovascular co-morbidities included hypertension, arrhythmia, coronary artery occlusion, and old stroke. Respiratory co-morbidities included chronic obstructive pulmonary disease and asthma. Treatment complications, defined as adverse events occurring within 30 d after operation or within the same hospitalization, were also assessed. Student's *t* test was used to analyze significant differences in clinical characteristics between the elderly and younger patient groups.

The meta-analyses were performed by STATA/SE 13.1 statistical software[6]. All analyzed data was treated as binary. Odds ratios and 95%CIs were computed from the binary data and used for the final meta-analyses. Both fixed and random effect models were calculated. Heterogeneity was assessed by the Cochrane *Q* test and was considered significant when $P < 0.10$. When there was substantially significant heterogeneity, a random effect model was used for meta-analysis. If heterogeneity was not significant, then a fixed effect model was used. In the meta-analysis, $P < 0.05$ was considered statistically significant. Publication bias was analyzed using funnel plot.

RESULTS

The prevalence of viral hepatitis comparing the young and elderly cohorts is shown in Table 1. The younger patient population was more likely to have positive HBsAg. The proportion of HCV Ab infection was comparable between the elderly and younger patients.

Surgical resection

Characteristics of patients who underwent surgical resection are shown in Table 2. Medical co-morbidities comparing the elderly *vs* younger patient populations are presented in Table 3. The elderly and younger patients were similar in respect to sex ratio, tumor size, cardiovascular complications, and diabetes. However, the elderly patients were more likely to have respiratory complications.

Overall survival: There were 9 studies which reported 1-year overall survival data, 13 studies which reported 3-year overall survival data, and 16 studies which reported 5-year survival data. After 1 year, the elderly group had significantly increased survival rates after surgical resection compared to the younger group (OR = 0.762, 95%CI: 0.583-0.994, $P = 0.045$). The meta-analysis demonstrated similar survival outcomes with surgical resection between the two groups (OR = 0.947, 95%CI: 0.777-1.154, $P = 0.67$ for the third year; and OR = 1.131, 95%CI: 0.895-1.430, $P = 0.304$ for the fifth year) (Figure 1). In terms of long term survival, the meta-analysis demonstrated no significant differences in outcomes of the elderly patients compared to younger patients, suggesting that surgical resection is a safe and effective treatment strategy for older patients. In the analysis of effects of overall survival at 1 year, there was no significant heterogeneity. There was substantial heterogeneity detected in the analysis of 5-year outcomes.

Disease free survival: The meta-analysis did not demonstrate any significant differences between the elderly and younger patients in terms of disease free survival after surgical resection (OR = 1.115, 95%CI:

Table 1 Prevalence of viral hepatitis among included studies

Study	Treatment	HBsAg positive (P = 0.023)		HCV Ab positive (P = 0.1681)	
		Young	Elderly	Young	Elderly
Yau *et al*[27]	SR/RFA/TACE	86.00%	52.00%	NA	NA
Chen *et al*[7]	SR	58.50%	28.60%	NA	NA
Hanazaki *et al*[8]	SR	23.70%	18.40%	40.70%	55.40%
Yeh *et al*[9]	SR	74.00%	25.80%	31.80%	63.20%
Ferrero *et al*[10]	SR	21.40%	10.90%	38.90%	60.90%
Kaibori *et al*[11]	SR	20.10%	9.70%	69.40%	71.60%
Oishi *et al*[4]	SR	23.70%	1.56%	66.00%	70.00%
Huang *et al*[12]	SR	88.80%	65.70%	1.10%	7.50%
Mirici-Cappa *et al*[13]	SR	15.00%	7.10%	47.10%	57.10%
Tsujita *et al*[14]	SR	23.00%	8.70%	70.60%	87.00%
Yamada *et al*[15]	SR	27.30%	36.30%	57.70%	54.50%
Nishikawa *et al*[16]	SR	17.50%	4.35%	56.30%	66.30%
Hirokawa *et al*[17]	SR	NA	NA	68.00%	50.00%
Ide *et al*[18]	SR	21.90%	12.50%	65.10%	68.80%
Liu *et al*[20]	SR	68.00%	33.00%	23.00%	36.00%
Kishida *et al*[21]	SR	31.00%	5.00%	43.00%	55.00%
Kim *et al*[22]	SR	64.50%	28.80%	7.80%	18.60%
Takahashi *et al*[24]	RFA	1.00%	6.80%	82.70%	93.40%
Mirici-Cappa *et al*[13]	RFA	13.00%	5.60%	52.20%	74.40%
Nishikawa *et al*[25]	RFA	11.30%	1.54%	73.90%	86.90%
Liu *et al*[20]	RFA	48.00%	43.00%	43.00%	39.00%
Fujiwara *et al*[26]	RFA	13.50%	3.70%	70.40%	79.60%
Yau *et al*[27]	TACE	87.00%	63.00%	10.00%	18.00%
Mirici-Cappa *et al*[13]	TACE	11.30%	12.10%	51.70%	59.90%
Liu *et al*[20]	TACE	52.00%	37.00%	35.00%	35.00%
Nishikawa *et al*[28]	TACE	15.50%	0.00%	58.30%	71.20%

TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; NA: Not available; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus.

Table 2 Preoperative data of patients with hepatocellular carcinoma who underwent surgical resection

Author	Year	Country	Design	Age cut off	Young, *n*	Elderly, *n*	Sex: male/female (P = 0.6947)		Child Pugh A/B		Tumor size (cm) (P = 0.984)	
							Y	E	Y	E	Y	E
Yau *et al*[27]	1999	China	Prospective	70	299	31	255/44	21/10	285/14	30/1	7.9	8.0
Wu *et al*[8]	1999	Taiwan	Retrospective	80	239	21	190/49	19/2	194/34	16/4	6.5	7.0
Hanazaki *et al*[8]	2001	Japan	Retrospective	70	283	103	222/61	71/32	226/56	76/27	NA	NA
Yeh *et al*[9]	2004	Taiwan	Prospective	70	398	34	310/88	27/7	152/199	12/18	6.7	5.5
Ferrero *et al*[10]	2005	Italy	Prospective	70	177	64	145/32	47/17	138/37	54/8	NA	NA
Kaibori *et al*[11]	2009	Japan	Retrospective	70	333	155	269/64	119/36	302/31	139/16	4.32	3.76
Oishi *et al*[4]	2009	Japan	Prospective	75	502	64	381/121	48/16	427/NA	59/NA	3.3	3.9
Huang *et al*[12]	2009	China	Retrospective	70	268	67	222/46	58/9	257/11	63/4	7.4	7.3
Mirici-Cappa *et al*[13]	2009	Italy	Retrospective	70	43	142	116/26	32/11	123/18	40/3	4.09	3.42
Tsujita *et al*[14]	2012	Japan	Prospective	80	385	23	253/132	15/8	264/121	18/5	3.2	3.5
Yamada *et al*[15]	2012	Japan	Prospective	80	267	11	205/62	6/5	245/22	9/2	4.8	5.2
Nishikawa *et al*[16]	2013	Japan	Prospective	75	206	92	161/45	61/31	198/8	90/2	4.8	4.6
Hirokawa *et al*[17]	2013	Japan	Retrospective	70	120	100	99/21	69/31	98/22	88/12	3.0	3.5
Ide *et al*[18]	2013	Japan	Retrospective	75	192	64	157/35	43/21	168/24	57/7	4.9	4.9
Taniai *et al*[19]	2013	Japan	Retrospective	75	353	63	271/82	39/24	265/84	56/7	NA	NA
Liu *et al*[20]	2014	Taiwan	Prospective	75	730	129	571/159	107/22	694/36	116/13	NA	NA
Kishida *et al*[21]	2015	Japan	Retrospective	75	82	22	66/16	20/2	NA	NA	3.0	3.5
Kim *et al*[22]	2015	South Korea	Retrospective	70	219	60	168/51	46/14	NA	NA	4.9	4.9

NA: Not available; E: Elderly; Y: Young.

0.815-1.359, P = 0.5488 for the first year; OR = 0.931, 95%CI: 0.730-1.189, P = 0.567 for the third year; and OR = 0.949, 95%CI: 0.748-1.205, P = 0.667 for the fifth year) (Figure 2). There was significant heterogeneity detected in the analysis of the effects of disease free survival rates.

Treatment complications: The meta-analysis did not demonstrate any significant differences between the elderly and younger patient groups in terms of treatment complications after surgical resection (OR = 1.082, 95%CI: 0.761-1.539, P = 0.659) (Figure 3). There was significant heterogeneity detected in the

Table 3 Co-morbidities of patients with hepatocellular carcinoma who underwent surgical resection

Author	Year	Country	CV co-morbidities (P = 0.341)		Resp co-morbidities (P = 0.031)		Diabetes (P = 0.086)		Alcohol (P = 0.737)	
			Young	Elderly	Young	Elderly	Young	Elderly	Young	Elderly
Yau *et al*[27]	1999	China	NA	NA	NA	NA	NA	NA	NA	NA
Chen *et al*[7]	1999	Taiwan	47.60%	12.60%	8.40%	14.30%	5.40%	14.30%	NA	NA
Hanazaki *et al*[8]	2001	Japan	11.00%	24.30%	7.40%	19.40%	8.50%	31.10%	NA	NA
Yeh *et al*[9]	2004	Taiwan	NA	NA	NA	NA	15.30%	38.20%	NA	NA
Ferrero *et al*[10]	2005	Italy	NA	NA	NA	NA	NA	NA	31.10%	20.30%
Kaibori *et al*[11]	2009	Japan	16%	39%	4%	16%	7%	23.00%	48.30%	35.40%
Oishi *et al*[4]	2009	Japan	NA	NA	NA	NA	NA	NA	NA	NA
Huang *et al*[12]	2009	China	NA	NA	NA	NA	NA	NA	NA	NA
Mirici-Cappa *et al*[13]	2009	Italy	NA	NA	NA	NA	NA	NA	12.90%	21.40%
Tsujita *et al*[14]	2012	Japan	11.90%	22.00%	13.00%	8.70%	29.40%	21.70%	NA	NA
Yamada *et al*[15]	2012	Japan	NA	NA	NA	NA	NA	NA	NA	NA
Nishikawa *et al*[16]	2013	Japan	13.10%	50.80%	10.70%	15.20%	33.50%	26.00%	NA	NA
Hirokawa *et al*[17]	2013	Japan	NA	NA	NA	NA	NA	NA	NA	NA
Ide *et al*[18]	2013	Japan	NA	NA	NA	NA	NA	NA	NA	NA
Taniai *et al*[19]	2013	Japan	NA	NA	NA	NA	NA	NA	NA	NA
Liu *et al*[20]	2014	Taiwan	NA	NA	NA	NA	NA	NA	15.00%	10.00%
Kishida *et al*[21]	2015	Japan	NA	NA	NA	NA	NA	NA	16.00%	18.00%
Kim *et al*[22]	2015	South Korea	NA	NA	NA	NA	13.70%	25.00%	3.20%	5.10%

NA: Not available.

analysis of the effects of complications. Among the most common complications were hemoperitoneum, bile leakage, intra abdominal abscess, and liver failure. Less common complications included ascites, pleural effusion, wound infection, prolonged jaundice, delirium, and renal failure.

Radio frequency ablation

Baseline characteristics and medical co-morbidities of HCC patients who underwent RFA are summarized in Table 4 and Table 5. Survival data of RFA between elderly and younger patients is shown in Table 6. The two populations were comparable in terms of tumor size, cardiovascular and respiratory co-morbidities, and diabetes. Elderly patients were more likely to be female. Younger patients were more likely to report alcohol use.

Overall survival: There were 4 studies which reported 1-year outcomes and 5 studies which reported 3-year, and 5-year overall survival data. The elderly group and younger group had similar survival outcomes for the first and third year after RFA (OR = 1.5, 95%CI: 0.788-2.885, P = 0.217 and OR = 1.352, 95%CI: 0.940-1.944, P = 0.104). For the fifth year, the elderly group had significantly worse survival rates compared to the younger group after RFA (OR = 1.379, 95%CI: 1.079-1.763, P = 0.01) (Figure 4). In the analysis of effects of overall survival, there was significant heterogeneity detected.

Treatment complications: The meta-analysis did not demonstrate any significant differences between the elderly and younger patient groups in terms of treatment complications after RFA (OR = 1.018, 95%CI: 0.522-1.987, P = 0.958) (Figure 5). There was no significant heterogeneity detected in the analysis of the effects of complications. Among the most common complications were hemoperitoneum, liver abscess, hemothorax, subcutaneous hematoma, and asymptomatic biloma[25,27]. Less common complications included pneumothorax, hemobilia, massive hepatic infarction, and gastrointestinal perforation[25,27].

TACE

Baseline characteristics of HCC patients who underwent TACE are summarized in Table 7. Survival data between elderly and younger patients who underwent TACE is shown in Table 8. The two populations were comparable in regards to sex ratio, tumor size, median survival in months, as well as 30 d mortality.

Overall survival: There were 5 studies which reported 1-year, 3-year, and 5-year overall survival data after TACE. The meta-analysis demonstrated significantly increased survival in the elderly group compared to the younger group for the first and third year (OR = 0.664, 95%CI: 0.548-0.805, P = 0 and OR = 0.795, 95%CI: 0.663-0.953, P = 0.013). At the fifth year, there were no significant differences in overall survival between the elderly group and younger group (OR = 1.256, 95%CI: 0.806-1.957, P = 0.313) (Figure 6). There was significant heterogeneity detected in the analysis of overall survival rates.

Treatment complications: The meta-analysis did not demonstrate any significant differences between the elderly and younger patient groups in terms of

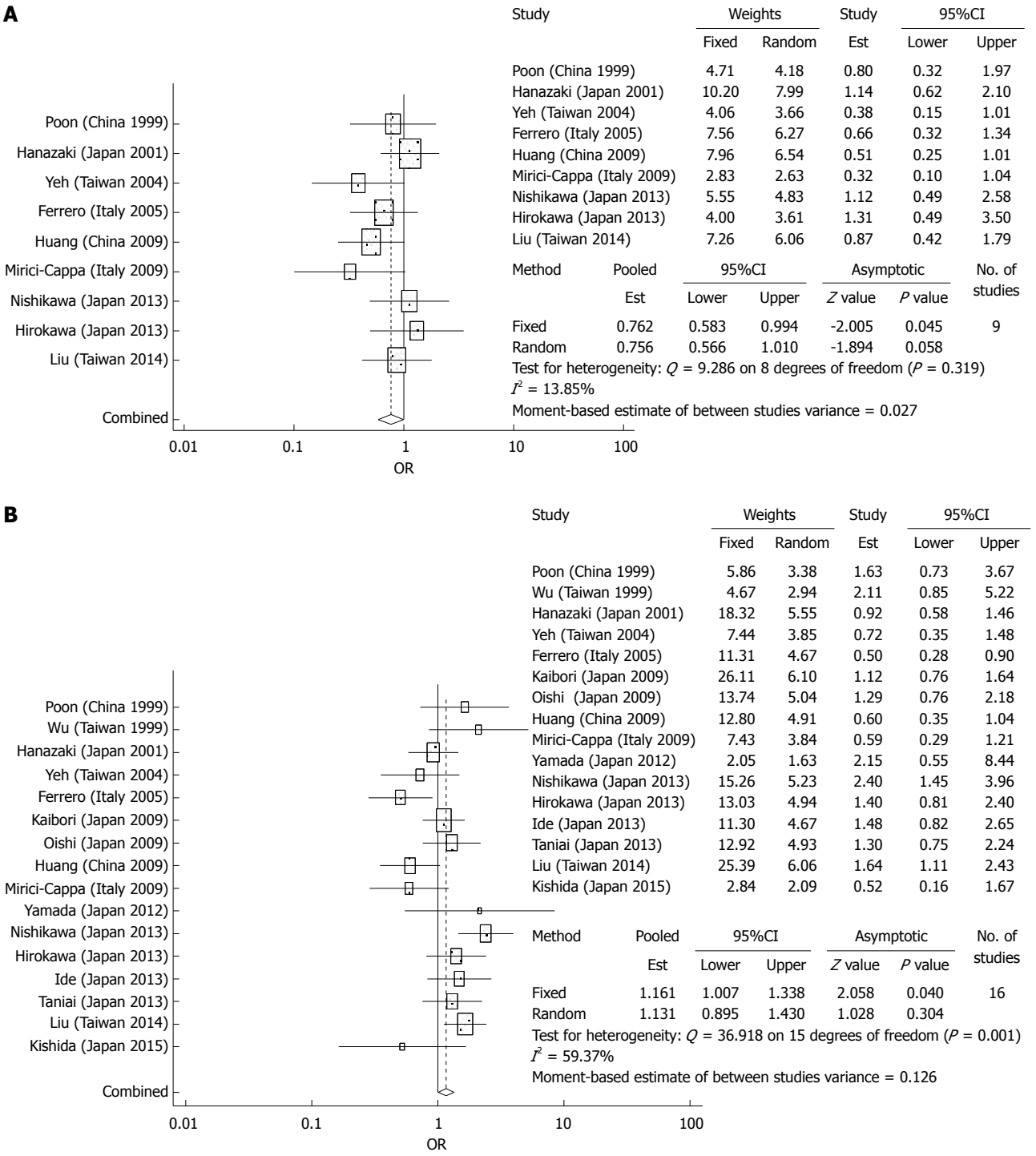
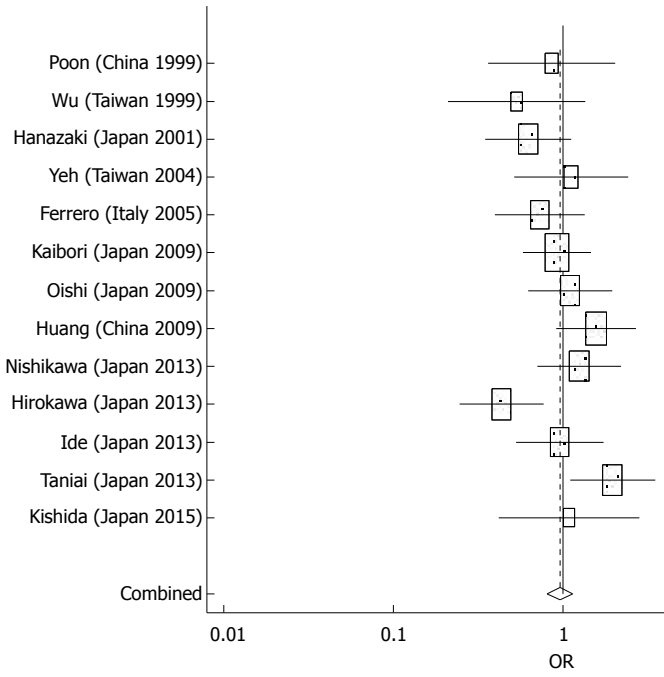


Figure 1 Forest plots for overall survival rates after surgical resection. A: Meta-analysis of 1-year overall survival outcomes; B: Meta-analysis of 5-year overall survival outcomes.

treatment complications after TACE (OR = 0.880 95%CI: 0.650-1.191, P = 0.407) (Figure 7). There was no significant heterogeneity detected in the analysis of the effects of complications. Among the most common complications were liver failure, liver abscess, peptic ulcers, and renal impairment[5,28,29]. Less common complications included acute pancreatitis, acute cholecystitis, gastrointestinal bleeding, and hepatic artery dissection[5,28,29]. No significant heterogeneity was detected in the analysis of effects of treatment complications.

Publication bias

Publication bias was assessed by symmetry of Begg's funnel plot (Figure 8). All the studies included had comparative data for 5-year overall survival. Level of symmetry was found to be high, indicating that there was no significant publication bias.



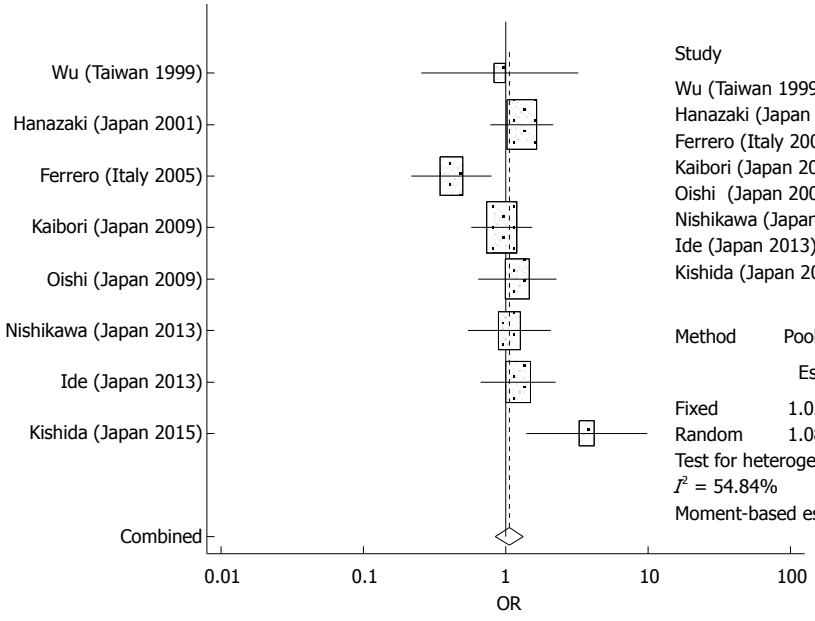
Study	Weights		Study	95%CI	
	Fixed	Random	Est	Lower	Upper
Poon (China 1999)	5.16	3.58	0.85	0.36	2.02
Wu (Taiwan 1999)	4.44	3.22	0.53	0.21	1.35
Hanazaki (Japan 2001)	11.19	5.72	0.62	0.35	1.12
Yeh (Taiwan 2004)	6.38	4.13	1.12	0.51	2.42
Ferrero (Italy 2005)	10.24	5.46	0.73	0.39	1.34
Kaibori (Japan 2009)	17.87	7.07	0.92	0.58	1.46
Oishi (Japan 2009)	11.86	5.89	1.10	0.62	1.95
Huang (China 2009)	13.15	6.19	1.56	0.91	2.68
Nishikawa (Japan 2013)	11.86	5.89	1.24	0.70	2.20
Hirokawa (Japan 2013)	11.86	5.89	0.43	0.25	0.77
Ide (Japan 2013)	10.81	5.62	0.96	0.53	1.74
Taniai (Japan 2013)	11.54	5.81	1.96	1.10	3.49
Kishida (Japan 2015)	4.21	3.09	1.09	0.42	2.82

Meta-analysis (exponential form)

Method	Pooled	95%CI		Asymptotic		No. of studies
	Est	Lower	Upper	*Z* value	*P* value	
Fixed	0.958	0.807	1.138	-0.488	0.626	13
Random	0.949	0.748	1.205	-0.430	0.667	

Test for heterogeneity: Q = 22.187 on 12 degrees of freedom (P = 0.035)
I^2 = 45.9%
Moment-based estimate of between studies variance = 0.086

Figure 2 Forest plot demonstrating meta-analysis of 5-year disease free survival after surgical resection.



Study	Weights		Study	95%CI	
	Fixed	Random	Est	Lower	Upper
Wu (Taiwan 1999)	2.38	1.80	0.91	0.26	3.24
Hanazaki (Japan 2001)	14.77	4.95	1.29	0.78	2.15
Ferrero (Italy 2005)	9.07	4.09	0.41	0.22	0.80
Kaibori (Japan 2009)	15.82	5.06	0.94	0.57	1.53
Oishi (Japan 2009)	9.62	4.20	1.20	0.64	2.26
Nishikawa (Japan 2013)	8.57	3.98	1.06	0.54	2.07
Ide (Japan 2013)	10.37	4.33	1.22	0.66	2.24
Kishida (Japan 2015)	4.03	2.61	3.70	1.39	9.83

Method	Pooled	95%CI		Asymptotic		No. of studies
	Est	Lower	Upper	*Z* value	*P* value	
Fixed	1.057	0.843	1.326	0.480	0.631	8
Random	1.082	0.761	1.539	0.441	0.659	

Test for heterogeneity: Q = 15.502 on 7 degrees of freedom (P = 0.030)
I^2 = 54.84%
Moment-based estimate of between studies variance = 0.134

Figure 3 Forest plot demonstrating meta-analysis of treatment complications of surgical resection.

DISCUSSION

As the prevalence of older patients with HCC has increased, there has been ongoing debate regarding appropriate treatment strategies for these patients. Multiple treatment options are available for HCC patients, with treatment allocation determined by multiple factors including patient clinical characteristics, tumor burden, and geographic variations in treatment expertise and utilization[7]. Our aim was to determine if age is an important contributor to patient survival after resection, ablation and chemoembolization. Given the relatively fewer number of patients eligible for liver transplantation in this population, this treatment strategy was not specifically evaluated in our study.

Surgical resection is a potentially curative option for the elderly patient. According to BCLC staging and treatment algorithm, surgical resection is indicated in

Table 4 Preoperative data of patients with hepatocellular carcinoma who underwent radiofrequency ablation

Author	Year	Country	Design	Treatment	Age cut off	Young, *n*	Elderly, *n*	Sex: male/female (*P* = 0.031)		Child Pugh A/B/C		Tumor size (cm) (*P* = 0.662)	
								Young	Elderly	Young	Elderly	Young	Elderly
Takahashi *et al*[24]	2010	Japan	Prospective	RFA	75	354	107	218/136	46/61	278/76	77/30	NA	NA
Mirici-Cappa *et al*[13]	2010	Italy	Retrospective	RFA or PEI	70	230	195	165/65	118/77	147/70/10	157/33/2	3.04	3.13
Nishikawa *et al*[25]	2012	Japan	Prospective	RFA	75	238	130	150/88	67/63	145/36/4	87/16	1.92	2.31
Liu *et al*[20]	2014	Taiwan	Prospective	RFA	75	336	147	221/115	96/51	80/17/3	89/11	NA	NA
Fujiwara *et al*[26]	2014	Japan	Retrospective	RFA	75	1048	353	705/343	191/162	782/255/11	287/63/3	2.40	2.50

RFA: Radiofrequency ablation; NA: Not available.

Table 5 Co-morbidities of patients with hepatocellular carcinoma who underwent radiofrequency ablation

Author	Year	Country	Design	Age cut off	Young, *n*	Elderly, *n*	Alcohol (*P* = 0.031)		CV comorbidities (*P* = 0.446)		Resp comorbidities (*P* = 0.474)		DM (*P* = 0.602)	
							Young	Elderly	Young	Elderly	Young	Elderly	Young	Elderly
Takahashi *et al*[24]	2010	Japan	Prospective	75	354	107	24.9%	8.4%	7.9%	13.1%	3.4%	9.3%	21.4%	16.8%
Mirici-Cappa *et al*[13]	2010	Italy	Retrospective	70	230	195	12.6%	5.6%	NA	NA	NA	NA	NA	NA
Nishikawa *et al*[25]	2012	Japan	Prospective	75	238	130	NA	NA	15.1%	34.6%	13.9%	25.4%	34.9%	28.5%
Liu *et al*[20]	2014	Taiwan	Prospective	75	336	147	19.0%	10.0%	NA	NA	NA	NA	NA	NA
Fujiwara *et al*[26]	2014	Japan	Retrospective	75	1048	353	15.9%	11.9%	NA	NA	NA	NA	NA	NA

NA: Not available.

Table 6 Survival data comparing elderly and younger patients undergoing radiofrequency ablation

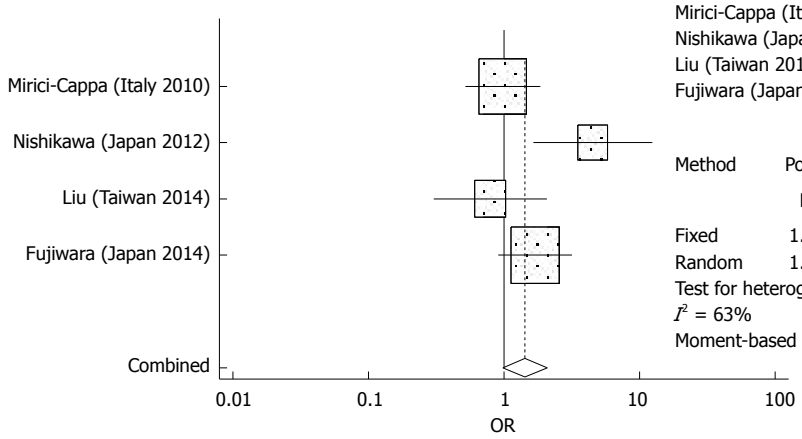
Author	Year	Young, *n*	Elderly, *n*	1-yr overall survival		3-yr overall survival		5-yr overall survival		*P* value
				Young	Elderly	Young	Elderly	Young	Elderly	
Takahashi *et al*[24]	2010	354	107	NA	NA	80.0%	82.0%	63.0%	61.0%	0.824
Mirici-Cappa *et al*[13]	2010	230	195	89.9%	90.1%	52.9%	53.4%	35.1%	29.0%	0.797
Nishikawa *et al*[25]	2012	238	130	97.6%	90.0%	83.7%	64.1%	64.0%	44.8%	0.001
Liu *et al*[21]	2014	336	147	95.0%	96.0%	81.0%	78.0%	65.0%	65.0%	0.690
Fujiwara *et al*[26]	2014	1048	353	97.3%	95.5%	82.3%	75.6%	62.9%	52.7%	< 0.001

NA: Not available.

HCC patients with a single tumor, PS 0, Child-Pugh class A, and no portal hypertension[31,32]. Among the studies analyzed in this meta-analysis, the elderly and younger patient populations did not differ significantly in terms of male: female sex ratio and tumor size. In terms of co-morbidities, the elderly patients had a higher proportion of respiratory disease. The proportion of cardiovascular disease and diabetes did not differ significantly between the two groups.

Our meta-analysis demonstrated that immediate surgical complications were also not significantly different between elderly and younger patients. In terms of long

A

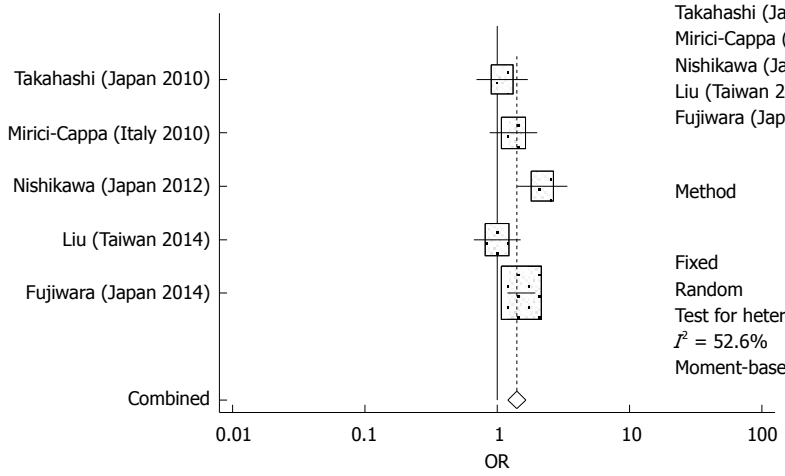


Study	Weights Fixed	Weights Random	Study Est	95%CI Lower	95%CI Upper
Mirici-Cappa (Italy 2010)	9.49	2.69	0.98	0.52	1.85
Nishikawa (Japan 2012)	3.78	1.88	4.52	1.65	12.39
Liu (Taiwan 2014)	4.17	1.98	0.79	0.30	2.07
Fujiwara (Japan 2014)	9.78	2.72	1.70	0.91	3.18

Method	Pooled Est	95%CI Lower	95%CI Upper	Asymptotic *Z* value	Asymptotic *P* value	No. of studies
Fixed	1.428	0.980	2.079	1.857	0.063	4
Random	1.500	0.788	2.855	1.234	0.217	

Test for heterogeneity: Q = 8.114 on 3 degrees of freedom (P = 0.044)
I^2 = 63%
Moment-based estimate of between studies variance = 0.266

B

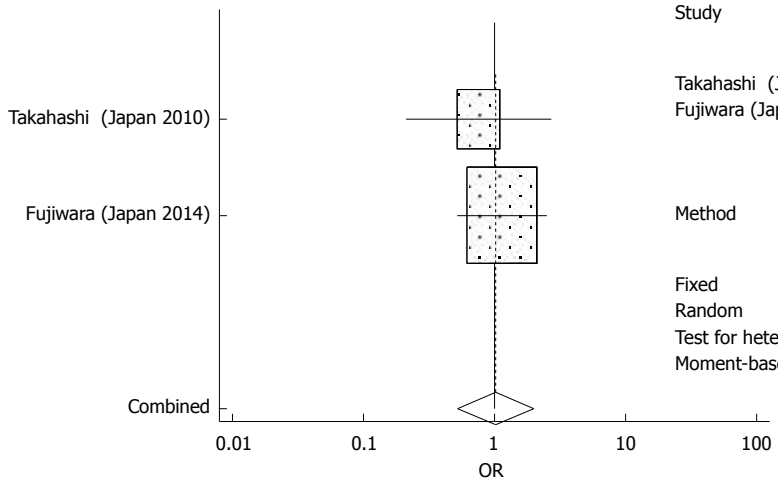


Study	Weights Fixed	Weights Random	Study Est	95%CI Lower	95%CI Upper
Takahashi (Japan 2010)	19.45	10.91	1.09	0.07	1.70
Mirici-Cappa (Italy 2010)	22.73	11.86	1.32	0.88	2.00
Nishikawa (Japan 2012)	20.27	11.16	2.19	1.42	3.39
Liu (Taiwan 2014)	23.26	12.01	1.00	0.67	1.50
Fujiwara (Japan 2014)	64.71	17.94	1.52	1.19	1.94

Method	Pooled Est	95%CI Lower	95%CI Upper	Asymptotic *Z* value	Asymptotic *P* value	No. of studies
Fixed	1.404	1.197	1.648	4.166	0.000	5
Random	1.379	1.079	1.763	2.569	0.010	

Test for heterogeneity: Q = 8.445 on 4 degrees of freedom (P = 0.077)
I^2 = 52.6%
Moment-based estimate of between studies variance = 0.040

Figure 4 Forest plots for overall survival rates after radiofrequency ablation. A: Meta-analysis of 1-year overall survival outcomes; B: Meta-analysis of 5-year overall survival outcomes.



Study	Weights Fixed	Weights Random	Study Est	95%CI Lower	95%CI Upper
Takahashi (Japan 2010)	2.36	2.36	0.76	0.21	2.71
Fujiwara (Japan 2014)	6.23	6.23	1.14	0.52	2.50

Method	Pooled Est	95%CI Lower	95%CI Upper	Asymptotic *Z* value	Asymptotic *P* value	No. of studies
Fixed	1.018	0.522	1.987	0.053	0.958	2
Random	1.018	0.522	1.987	0.053	0.958	

Test for heterogeneity: Q = 0.289 on 1 degrees of freedom (P = 0.591)
Moment-based estimate of between studies variance = 0.000

Figure 5 Forest plot for treatment complications after radiofrequency ablation.

Table 7 Preoperative data of patients undergoing transarterial chemoembolization

Author	Year	Country	Design	Tx	Age cut off	Young, *n*	Elderly, *n*	Sex: male/female (*P* = 0.272)		Child Pugh A/B/C		Tumor size (cm) (*P* = 0.953)	
								Young	Elderly	Young	Elderly	Young	Elderly
Yau *et al*[27]	2009	China	Prospective	TACE	70	317	67	272/45	54/13	262/55	52/15	8.70	8.50
Yau *et al*[27]	2009	China	Prospective	TACE	70	843	197	715/128	143/54	674/159/10	165/32	NA	NA
Mirici-Cappa *et al*[13]	2010	Italy	Retrospective	TACE	70	396	158	301/95	117/41	234/135/21	113/40/3	3.89	3.86
Liu *et al*[20]	2014	Taiwan	Prospective	TACE	75	604	271	446/158	222/49	72/22/3	86/14	NA	NA
Nishikawa *et al*[28]	2014	Japan	Prospective	TACE	75	84	66	63/21	34/32	52/32	53/13	5.10	5.70

TACE: Transarterial chemoembolization; NA: Not available.

Table 8 Survival data comparing elderly and younger patients undergoing transarterial chemoembolization

Author	Age cut off	Young, *n*	Elderly, *n*	Median survival (mo) (*P* = 0.683)		Post Op complication (*P* = 0.854)		30-d mortality (*P* = 0.698)		1-yr overall survival		3-yr overall survival		5-yr overall survival		*P* value
				Y	E	Y	E	Y	E	Y	E	Y	E	Y	E	
Yau *et al*[27]	70	317	67	9	12	26.0%	24.0%	5.0%	7.0%	41.0%	53.0%	18.0%	25.0%	13.0%	17.0%	0.277
Yau *et al*[27]	70	843	197	8.1	14	26.9%	24.4%	3.5%	4.7%	39.2%	54.4%	14.9%	23.2%	8.4%	10.6%	< 0.003
Mirici-Cappa *et al*[13]	70	396	158	27	26	NA	NA	NA	NA	78.9%	79.4%	32.0%	36.4%	13.3%	6.4%	0.730
Liu *et al*[20]	75	604	271	NA	NA	NA	NA	NA	NA	79.0%	84.0%	57.0%	57.0%	42.0%	39.0%	0.953
Nishikawa *et al*[28]	75	84	66	29.3	34.8	6.0%	5.0%	0.0%	0.0%	78.2%	84.1%	39.3%	48.0%	33.8%	15.0%	0.887

Y: Young; E: Elderly; NA: Not available.

term outcomes, overall survival was similar between elderly and younger patients. Huang *et al*[12] reported that elderly patients actually had a significantly increased overall survival rate compared to non elderly patients[13]. Huang also found lower hepatitis B surface antigen positive rate, higher hepatitis C virus infection rate, and more preoperative co-morbidities in the elderly population. Hirokawa *et al*[17] found that disease free survival was worse in elderly patients compared to non elderly patients after surgical resection[18]. Like in many other studies with elderly patients and HCC, they found a high prevalence of HCV infection[18]. Disease free survival was stratified to response to interferon in HCV-infected patients and the study showed that responders to IFN had significantly increased survival to non responders. Generally, elderly patients have had poor response to interferon therapy due to severe side effects. Improvements of antiviral therapy with the newer oral agents could possibly lead to improvements in disease free survival in elderly HCC patients with HCV infection.

Radiofrequency ablation has become an increasingly popular option for the elderly HCC patient[14]. The AASLD and EASL guidelines present RFA as a potential treatment option for compensated cirrhotic patients with good functional status and lesions less than 5 cm in size[31,32]. There have not been many studies investigating the outcomes of RFA in elderly patients. In our analysis of preoperative patient characteristics, we found that there was a higher proportion of females in the elderly population and a higher proportion of alcohol use in the younger patient population. The proportion of diabetes, cardiovascular disease, and respiratory disease were similar in both patient groups. Our meta-analysis demonstrated no significant differences in 1-year and 3-year overall survival in elderly and non elderly patients who underwent RFA. At 5 years, the younger had significantly better clinical outcomes. As the data regarding long term outcomes of elderly patients with RFA continues to be lacking, the meta-analysis is limited by a small number of studies and showed significant heterogeneity. Similar to our findings, Fujiwara *et al*[26] also showed lower mortality at 5 years in the elderly patients. However, an additional risk analysis demonstrated that there was a significant difference in liver-unrelated death between

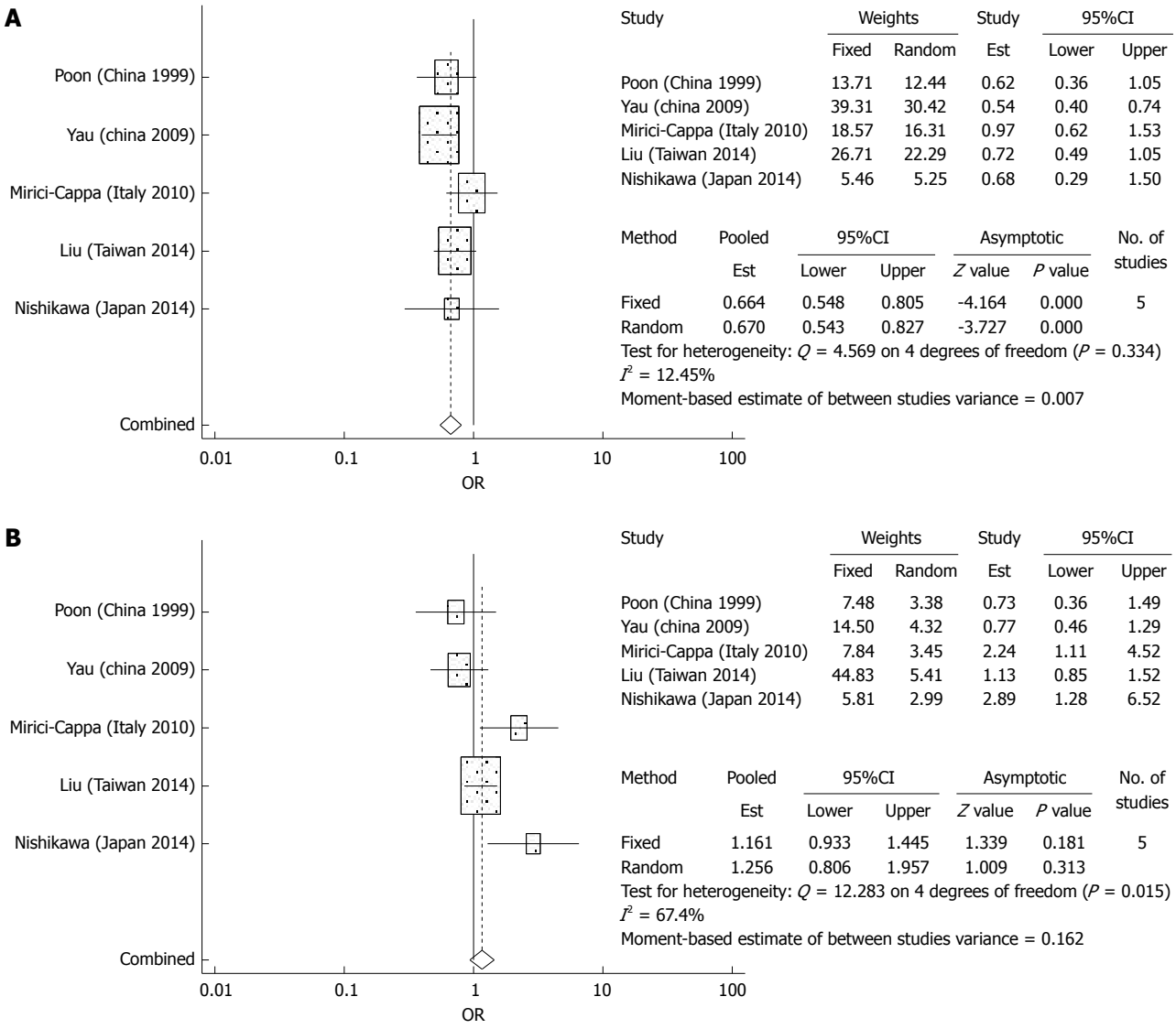


Figure 6 Forest plots for overall survival rates after transarterial chemoembolization. A: Meta-analysis of 1-year overall survival outcomes; B: Meta-analysis of 5-year overall survival outcomes.

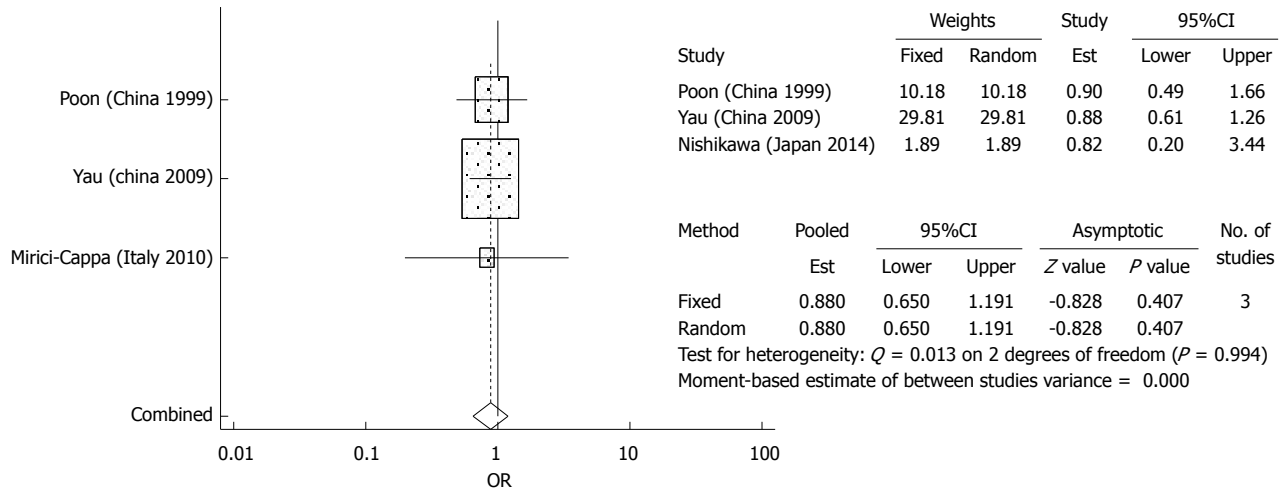


Figure 7 Forest plot for treatment complications after transarterial chemoembolization.

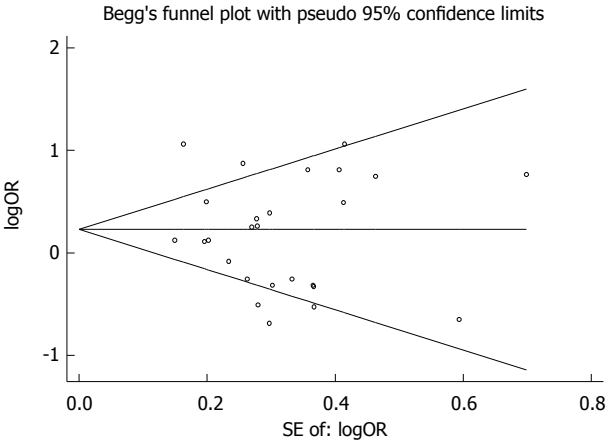


Figure 8 Begg's funnel plot with standard error as the X axis and the log of the odds ratio as the Y axis.

the elderly and younger patients[26]. This suggested that elderly patients tended to die from liver-unrelated causes at 5 years. Nishikawa *et al*[25] demonstrated decreased cumulative overall survival and disease free survival at 1-, 3-, and 5-year intervals in the elderly patient group[26]. The results in this study were poorer compared to other reports. Both Nishikawa *et al*[25] and Fujiwara *et al*[26] did not demonstrate any significant differences in terms of duration of hospitalization and serious adverse events, suggesting that RFA is a safe procedure for elderly patients. Although limited by a small number of studies, our meta-analysis demonstrated no significant difference in treatment complications among the elderly patients and their younger counterparts.

Transarterial chemoembolization is recommended for unresectable, Child-Pugh class A or B patients with multiple HCC with no vascular invasion. In terms of treatment complications, we found that there was no significant difference between the elderly patients and their younger counterparts. Our meta-analysis demonstrated increased overall survival in the elderly patients at 1-year and 3-year intervals. At 5 years, there were no significant differences in clinical outcomes between the elderly and younger patients. There was no significant difference in postoperative complications and 30 d mortality between the two groups. A study by Mondazzi *et al*[29] demonstrated that the independent prognostic factors affecting survival in elderly patients undergoing TACE were tumor stage, tumor markers, and hepatic functional reserve. Advanced age was not shown to be an adverse predictor of decreased survival[30]. Our TACE meta-analysis is also limited by a small number of studies and heterogeneity. The largest study conducted by Yau *et al*[27] showed increased overall median survival and disease-specific survival in elderly patients compared to younger patients. There are only a limited number of studies on the clinical outcomes of TACE in the elderly. In the studies that were analyzed, co-morbidities of the elderly patients including cardiovascular disease, respiratory disease, and diabetes were not included. Further investigation is warranted, but given the limited data, TACE also appears to be a safe treatment option for elderly HCC patients.

This meta-analysis suggests that age should not be a negative factor when considering treatment allocation and outcomes given equivalent survival and complications with resection, ablation, and TACE between younger and older patients. In the largest cohort of elderly HCC patients published, Liu *et al*[20] used propensity score analysis system to match elderly and younger HCC patients and similarly did not find an association with advanced age and inferior long-term survival for HCC patients receiving surgical resection, RFA, and TACE. In that study, 74% of younger patients received curative treatment as the initial treatment, as compared to 67% of elderly patients. Elderly patients were less likely to undergo surgical resection and instead received RFA or TACE compared to younger patients. The elderly patients were more likely to have compensated cirrhosis, lower AFP, fewer tumors with smaller tumor volume, and less vascular invasion but were more likely to advanced BCLC staging. It may seem contradictory that elderly HCC patients tend to have more advanced BCLC grading, but less advanced cancer. This may be explained by the incorporation of performance status in the BCLC grading system as the elderly patients did have poorer performance status compared to their younger counterparts. Advanced BCLC stage could also account for less resection. In this study, age was not found to be an independent predictor of poor prognosis. Thus, when deciding the optimal treatment strategy for elderly HCC patients, thorough evaluation based on cancer stage and general condition is important.

The majority of the studies included in the meta-analysis were done in eastern Asia, predominantly Japan and China. The clinical outcomes of elderly HCC patients in the western world, particularly in the United States, have not been thoroughly investigated. There is an urgent need for further investigation as HCC is becoming a global health problem. In the United States, reports suggest that HCC incidence peaks above the age of 70[2]. Most of the existing literature regarding elderly HCC patients pertains to chronic hepatitis B or C. In the western world, there is an increasing group of elderly HCC patients with non alcoholic fatty liver disease. There is limited data regarding the optimal treatment strategy of this group of patients as they also tend to have multiple co-morbidities. To increase external validity, further studies from western countries are desperately needed as the Asia Pacific region is a highly hepatitis B endemic area.

Several limitations to this meta-analysis exist. Studies included are observational cohorts by design given the lack of randomized controlled trials for HCC treatments. The multiple studies included in this review

contain diverse patient demographic and geographic features resulting in significant heterogeneity. Few studies investigated the safety and validity of surgical resection, TACE, and RFA for elderly patients with HCC. Regarding other therapeutic options such as molecular-targeted therapy such as sorafenib, radioembolization, and liver transplantation, there is even less data on clinical outcomes in elderly HCC patients and therefore these modalities were not included in our analysis.

In conclusion, this meta-analysis suggests that surgical resection, TACE, and RFA are safe and effective treatment options for elderly patients with HCC. Overall, elderly patients have similar success with these treatments compared to younger patients and should be considered for all treatments pending their clinical status and cancer burden.

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COMMENTS

Background

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, has become the fifth most common cancer and the third most common cause of cancer-related mortality worldwide. The management of HCC is multi-disciplinary with a wide range of treatment options ranging from liver resection, liver transplantation, locoregional therapies including ablation and transarterial chemoembolization (TACE), and molecular-targeting therapies. As our society continues to age, there will be an increasing proportion of elderly patients with hepatocellular carcinoma. The optimal management strategy for elderly patients with HCC remains controversial.

Research frontiers

There have been several studies done on elderly patients with hepatocellular carcinoma, comparing their clinical outcomes to non-elderly patients. However, these studies are often limited by small sample size or heterogeneity. This is the first meta-analysis and systematic review to combine data from the multiple studies, in attempt to shed light on the safety and efficacy of various treatment modalities for hepatocellular carcinoma in the elderly population.

Innovations and breakthroughs

Based on this meta-analysis, elderly patients with hepatocellular carcinoma have similar outcomes compared to non-elderly patients. Specifically, they have similar three year survival after resection and ablation and an improved three year survival after TACE, compared to non-elderly patients. At five years, elderly patients had a lower survival after ablation but similar survival with resection and TACE as compared to younger patients. Heterogeneity of patient populations and selection bias can explain some of these findings. Overall, elderly patients have good success with these treatments and should be considered for all treatments after assessment of their clinical status and cancer burden.

Applications

The findings from this meta-analysis can be used to guide the treatment approach of elderly patients with hepatocellular carcinoma. The analysis included clinical outcomes of locoregional therapies including surgical resection, chemoembolization, and radiofrequency ablation. The studies included were limited by heterogeneity with diverse patient demographic and geographic features. Further investigation is warranted regarding other therapeutic options such as molecular-targeted, radioembolization, and liver transplantation.

Terminology

TACE is a minimally invasive procedure which involves injecting particles coated with chemotherapeutic agents into a tumor's arterial supply. Radiofrequency ablation (RFA) is another minimally invasive procedure in which rapidly alternating current and electricity is delivered to a tumor to induce necrosis.

Peer-review

The aim of this meta-analysis was to investigate the clinical outcomes of surgical resection and locoregional treatments for HCC in elderly patients defined as aged 70 years or more. The authors suggest that surgical resection, TACE, and RFA are safe and effective treatment options for elderly patients with HCC. The elderly patients have similar success with these treatments compared to younger patients and should be considered for all treatments pending their clinical status and cancer burden. This manuscript is important as it will help to shed light on the management of HCC in the elderly population.

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