ISSN 1948-5182 (online)

World Journal of *Hepatology*

World J Hepatol 2015 August 18; 7(17): 2053-2126

Published by Baishideng Publishing Group Inc

World Journal of Hepatology

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

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2014-2017

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World Journal of Hepatology

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NAME OF JOURNAL
World Journal of Hepatology

ISSN
ISSN 1948-5182 (online)

LAUNCH DATE
October 31, 2009

FREQUENCY
36 Issues/Year (8th, 18th, and 28th of each month)

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PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
August 18, 2015

INSTRUCTIONS TO AUTHORS
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DOI: 10.4254/wjh.v7.i17.2053

World J Hepatol 2015 August 18; 7(17): 2053-2057
ISSN 1948-5182 (online)

EDITORIAL

Second line systemic therapies for hepatocellular carcinoma: Reasons for the failure

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Author contributions: All authors contributed to writing the paper and had full control over preparation of manuscript; all authors approved the final draft manuscript.

Conflict-of-interest statement: We declare that we have no proprietary, financial, professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Second line systemic therapies for hepatocellular carcinoma: Reasons for the failure".

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Received: February 23, 2015
Peer-review started: February 25, 2015
First decision: June 3, 2015
Revised: June 22, 2015
Accepted: June 30, 2015
Article in press: July 2, 2015
Published online: August 18, 2015

Abstract

Hepatocellular carcinoma (HCC) is the main cause of death in patients with cirrhosis, with an increasing incidence worldwide. Sorafenib is the choice therapy for advanced HCC. Over time several randomized phase Ⅲ trials have been performed testing sunitinib, brivanib, linifanib and other molecules in head-to-head comparison with Sorafenib as first-line treatment for advanced-stage HCC, but none of these has so far been registered in this setting. Moreover, another feared vacuum arises from the absence of molecules registered as second-line therapy for patients who have failed Sorafenib, representing an urgent unmet medical need. To date all molecules tested as second-line therapies for advanced hepatocellular carcinoma, failed to demonstrate an increased survival compared to placebo. What are the possible reasons for the failure? What we should expect in the near future?

Key words: Systemic therapies; Sorafenib; Barcelona Clinic Liver Cancer; Hepatocellular carcinoma

Core tip: Hepatocellular carcinoma (HCC) is the main cause of death in patients with cirrhosis with an increasing incidence worldwide. Sorafenib is the choice therapy for advanced HCC. Since then no other molecule has been registered as first-line therapy in this setting and one more vacuum arises from the absence of molecules registered as second-line therapy for patients who have failed Sorafenib. What are the reasons and what we should expect in the near future?

Maida M, Iavarone M, Raineri M, Cammà C, Cabibbo G. Second line systemic therapies for hepatocellular carcinoma: Reasons for the failure. *World J Hepatol* 2015; 7(17): 2053-2057 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2053.

htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2053

TEXT

Hepatocellular carcinoma (HCC) is the main cause of death in patients with cirrhosis with an increasing incidence worldwide and a poor prognosis even when treatments have been considered as potentially radical[1,2].

The natural history of this tumour is severe and extremely heterogeneous, due to the complex interplay between its biological characteristics and the frequent presence of an underlying chronic liver disease[3,4]. As part of this biological and clinical heterogeneity, several lines of evidence based on microarray technology point out how heterogeneity can be explained, least in part, from the identification of several molecular signatures (WNT, TGFβ, MAPK, EGFR, IGF-R, MET/HGF) able to predict prognosis and survival of HCC patients[5-8]. In this regard, a recent work remarked on the importance of genetic predisposition testing, in a clinical setting, a five-gene hepatic transcriptomic signature (angiopoietin-2, NETO2, DLL4, ESM1, NR4A1) able to identify patients with extremely rapid tumour growth and ominous prognosis[9].

In the absence of an ideal prognostic model, treatment algorithms for patients with HCC in Europe and North America have been assessed on the basis of the Barcelona Clinic Liver Cancer (BCLC) staging classification for HCC. It is currently the only staging system that includes an integrated assessment of liver disease, tumor extension, and presence of constitutional symptoms, providing in the meantime an indication of the first-line treatment. It classify stages of disease into five subgroups, from 0 to D, each associated with a specific therapy and prognosis[10].

As well known, the worst prognosis is allocated to patients with end stage disease (BCLC D). They cannot benefit from any specific cancer therapy due to the poor life expectance (median survival less than 3 mo), and could only receive the best available supportive care.

Besides this, patients classified as advanced stage (BCLC C) have a better prognosis of the above, but sill represents a critical group of the whole HCC population. In this subset surgical or loco-regional therapies are contraindicated and systemic therapies remains the only treatment option.

Previously, no effective therapy was offered for the treatment of patients at this stage, a scenario that was partially subverted in 2007 by the advent of Sorafenib, an oral multikinase inhibitor that, by blocking cell proliferation and angiogenesis of the tumour, has shown an improvement of survival according to two pivotal randomized controlled trials (RCTs)[11,12].

What happened next, up to now? About eight years after its introduction Sorafenib has then certainly innovated the clinical scenario of HCC, providing a practical treatment option in a subset of patients, which until then could not benefit from any therapy, but ultimately it has not represented the best desirable therapeutic progress for advanced HCC. Some lines of evidences have attenuated the effectiveness of Sorafenib and its safety profile in clinical practice compared to with those reported in the pivotal trials. Data from a field practice prospective study in Italy, Sorafenib Italian Assessment (SOFIA), confirmed the efficacy of Sorafenib with a lower safety profile compared to that of the phase Ⅲ trial, showing also a significant proportion of patients who required a dose adjustment with an increased survival rate in those patients who received dose-adjusted Sorafenib (400 mg daily) for ⩾ 70% of the treatment period, due to adverse events (AEs) or comorbidities, compared to those that received a full-dosed regimen (800 mg daily)[13]. Moreover the cost-effectiveness analysis based from the SOFIA study, showed that dose-adjusted Sorafenib therapy, compared to full-dose, is a cost-effective treatment for advanced HCC[14].

What came out after Sorafenib era? To date, as for the "first-line" scenario, different drugs have been tested, two different trial designs have been adopted for advanced HCC. The first one was the head-to-head comparison with Sorafenib, which is generally applied only if the effectiveness of a new agent shown to be very promising in early-phase trials.

Over time several randomized phase Ⅲ trials have been performed testing sunitinib, brivanib, linifanib and other molecules in head-to-head comparison with Sorafenib as first-line treatment for advanced-stage HCC, but none of these has so far been registered in this setting[15-18]. Is important to note that, despite their safety and efficacy unfavorable results, many of these phase Ⅲ trials were designed with the purpose of demonstrating the non-inferiority on Sorafenib. Anyway, non-inferiority studies have no ethical foundation, since they do not guarantee any possible advantage to patients and only favour pharmaceutical companies' interests. For these reasons, non-inferiority trials in oncology should be avoided, especially when testing first-line therapies[15,19].

A second modality for first line therapy is to test a new drug in combination with Sorafenib *vs* Sorafenib alone. This modality has been adopted in different RCTs but failed to show a benefit in term of survival, and non of these combinations has been registered for advanced HCC.

In conclusion, Sorafenib remains the only drug for patients with advanced HCC, and dose-tailored to AEs and comorbidities, appears the only therapeutic innovation with Sorafenib.

Moreover, another feared vacuum arises from the absence of molecules registered as second-line therapy for patients who failed Sorafenib. In fact, in the last years, three randomized phase Ⅲ trials testing brivanib, everolimus and ramucirumab as second-line therapies for advanced hepatocellular carcinoma, failed to demonstrate

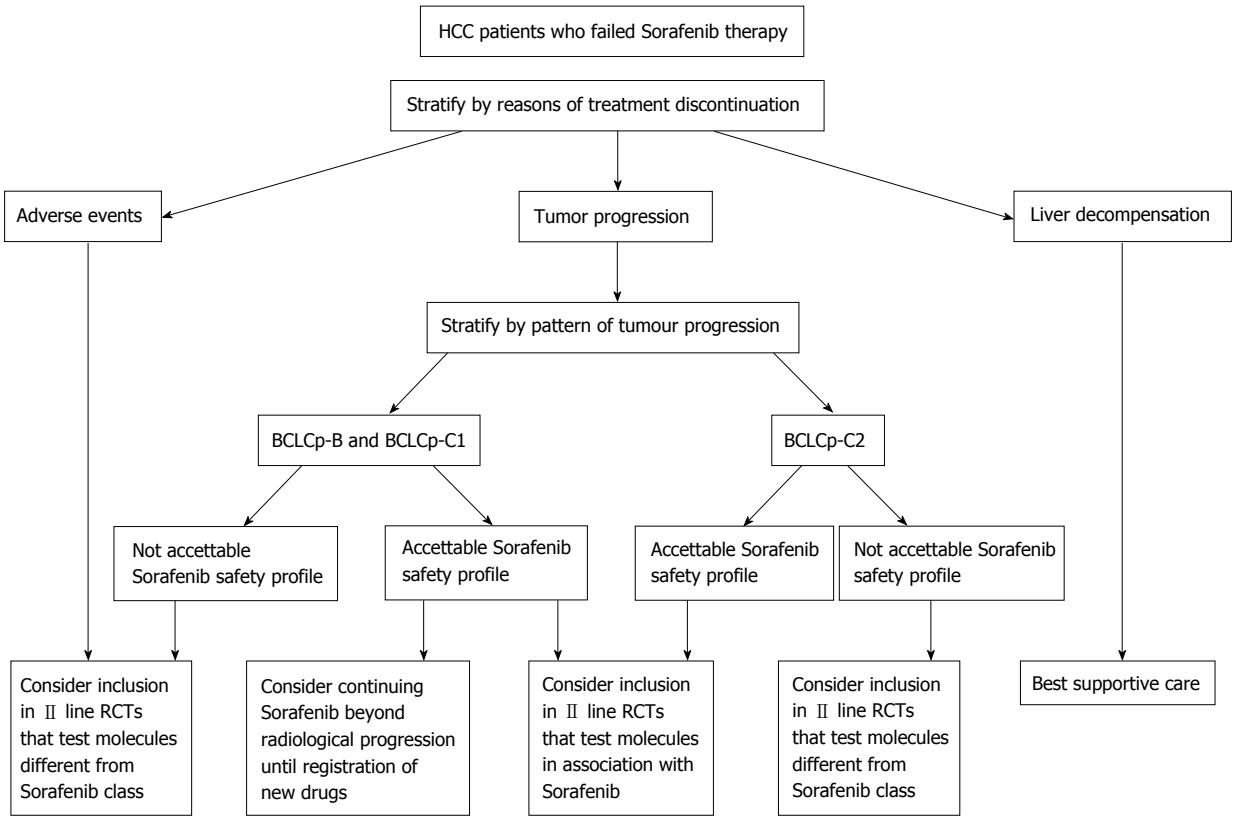

Figure 1 Proposed algorithm for the management of the hepatocellular carcinoma patients who discontinue permanently Sorafenib, combining the results of studies evaluating reason of Sorafenib discontinuation of Iavarone *et al*[23] and Reig *et al*[22]. HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; RCTs: Randomized controlled trials.

an increased survival compared to placebo[20-22]. Following these failures and from clinical practice, we have learned that patients who failed Sorafenib therapy represent a fragile and extremely heterogeneous population from which emerges a complex prognosis.

In this line, a study by Reig *et al*[23] clearly demonstrated a substantial differences in survival rates among progressors during Sorafenib therapy related to the pattern of HCC progression. The study shows a worse prognosis for patients developing new extrahepatic tumour lesions compared to those with expanding pre-existing lesions or new intra-hepatic nodules, only. While post progression survival of patients under Sorafenib is driven by tumour progression pattern, less known are the factors able to affect prognosis when therapy is discontinued due to other reasons.

In this regard, a recent study has been performed with the aim to identify predictors of survival on a sample of two-hundred and sixty HCC patients who discontinued Sorafenib therapy for any reasons[24]. Overall median post Sorafenib survival (PSS) was 4.1 mo, while median PSS was 9.3, 4.6 and 1.6 mo for BCLC B, BCLC C and BCLC D stage, respectively. Performance status (PS) (HR = 2.4), prothrombin time (HR = 2.9), macrovascular invasion (HR = 1.8), extrahepatic spread (HR = 1.6), alpha-fetoprotein $\geqslant$ 400 ng/mL (HR = 1.4) and reason for Sorafenib discontinuation were find to be independent predictors of worse survival by multivariate analysis. Between all causes for Sorafenib interruption the best prognosis was assessed for patients who interrupted for AEs, followed by tumour progression and then by liver function worsening group (liver decompensation *vs* AEs HR = 2.6, tumor progression *vs* AEs HR = 1.5).

Within the whole court, 200 patients (77%) with Child-Pugh score up to 7, were considered eligible for inclusion in second-line experimental therapy. In this subset, the presence of macrovascular invasion and extrahepatic spread, PS and the reason for Sorafenib discontinuation, were found to be independent predictors of mortality by multivariate analysis.

Therefore discontinuation due to adverse events in the absence of PS impairment and vascular or extrahepatic diffusion of the tumor, estimates the best post Sorafenib survival in compensated patients, emphasizing the role of these predictors in stratifying patients potentially eligible for second-line studies[24].

This adds further weight to the need to change the current design of second-lines trials, focusing on the importance of stratification among the clinical and biological heterogeneity of cancer after exposure to first-line systemic therapy.

This, in the near future, the genetic signature will likely provide a great contribution for prognostic profiling of patients with advanced HCC and then for a proper planning and design of first and second line clinical trials. In this line, a recent multicentric, randomised, placebo-controlled, double-blind, phase 2

study testing Tivantinib, a selective oral inhibitor of MET, *vs* placebo, as second-line therapy for advanced HCC, showed that, regardless of treatment, patients with MET high-expression tumours had significantly shorter overall survival compared to the MET low-expression subgroup[25].

Waiting for new effective therapies and further advances in genetic prognostic characterization of the tumor, the evidence that PSS depends on the reasons of therapy discontinuation could support clinicians in counselling and management of these patients.

In this line, patients who discontinue therapy for adverse events may be considered for inclusion in Ⅱ line RCTs that test molecules different from Sorafenib class. The same way as these, can be managed those patients who discontinue therapy for tumor progression with a poor experienced Sorafenib safety profile.

Contrariwise, another strategy that could be offered to patients with radiological progression and good Sorafenib safety profile, is to continue Sorafenib therapy until symptomatic progression, or to consider inclusion in Ⅱ line RCTs that test molecules in association with Sorafenib. In this group of progressor patients, the decision making process, could be supported by stratification using "BCLC staging system upon progression". On the contrary the patients who suspend for hepatic failure may only receive the best supportive care, since they have a poor prognosis (Figure 1).

In conclusion, it is clear by now, especially from the clinical point of view, the importance of a correct identification of the reason for Sorafenib discontinuation, in order to obtain an optimal management of HCC patients.

On the other hand, despite this and the proposed strategy, we are still facing with a scenario showing us the failure of the of first and second line systemic therapy trials, leaving Sorafenib as the last outpost for the treatment of advanced stage, a picture almost unchanged over the past seven years. What to expect from the future?

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P- Reviewer: Chen CH, Du Z, Snyder N **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4254/wjh.v7.i17.2058

World J Hepatol 2015 August 18; 7(17): 2058-2068
ISSN 1948-5182 (online)

EDITORIAL

Intestinal barrier dysfunction in cirrhosis: Current concepts in pathophysiology and clinical implications

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Author contributions: Tsiaoussis GI and Thomopoulos KC wrote the paper, conceptualized and designated the work; Assimakopoulos SF, Tsamandas AC and Triantos CK critically revised the paper.

Conflict-of-interest statement: None of the authors have any conflict of interest related to this work.

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Received: February 25, 2015
Peer-review started: February 26, 2015
First decision: May 14, 2015
Revised: June 22, 2015
Accepted: June 30, 2015
Article in press: July 2, 2015
Published online: August 18, 2015

Abstract

The intestinal lumen is a host place for a wide range of microbiota and sets a unique interplay between local immune system, inflammatory cells and intestinal epithelium, forming a physical barrier against microbial invaders and toxins. Bacterial translocation is the migration of viable or nonviable microorganisms or their pathogen-associated molecular patterns, such as lipopolysaccharide, from the gut lumen to the mesenteric lymph nodes, systemic circulation and other normally sterile extraintestinal sites. A series of studies have shown that translocation of bacteria and their products across the intestinal barrier is a commonplace in patients with liver disease. The deterioration of intestinal barrier integrity and the consulting increased intestinal permeability in cirrhotic patients play a pivotal pathophysiological role in the development of severe complications as high rate of infections, spontaneous bacterial peritonitis, hepatic encephalopathy, hepatorenal syndrome, variceal bleeding, progression of liver injury and hepatocellular carcinoma. Nevertheless, the exact cellular and molecular mechanisms implicated in the phenomenon of microbial translocation in liver cirrhosis have not been fully elucidated yet.

Key words: Cirrhosis; Intestinal barrier; Tight junction; Bacterial translocation; Intestinal bacterial overgrowth

Core tip: Intestinal barrier function is impaired in patients with cirrhosis and this derangement seems to be associated with liver disease severity. This phenomenon is multifactorial and the exact pathophysiological mechanisms which are implicated in this deterioration have not been fully elucidated yet. The disruption of intestinal barrier integrity and the subsequent increased

intestinal permeability in cirrhotic patients promote bacterial translocation and play a major role in the development of severe clinical complications affecting natural history of liver disease and patients' survival.

Tsiaoussis GI, Assimakopoulos SF, Tsamandas AC, Triantos CK, Thomopoulos KC. Intestinal barrier dysfunction in cirrhosis: Current concepts in pathophysiology and clinical implications. *World J Hepatol* 2015; 7(17): 2058-2068 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2058.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2058

INTRODUCTION

Cirrhosis and portal hypertension associated complications are a common cause of mortality worldwide. Increased intestinal permeability and subsequent bacterial translocation to the mesenteric lymph nodes and extraintestinal sites are well established in these patients[1,2]. Endotoxemia seems to be a key factor and results in a cascade of immunomodulatory, cellular and molecular events. Potential mechanisms that can promote BT are intestinal bacterial overgrowth (IBO) and gut flora disturbances, increased intestinal permeability *via* the paracellular and intracellular route and local as well as systemic immune dysfunction[3-7]. Cirrhosis is also associated with increased oxidative stress in the systematic circulation, the intestinal and liver tissue, which in turn acts as a harmful agent to the intestinal epithelial cells, affects apoptosis and cellular proliferation, deteriorates the expression of tight junction (TJ) proteins and favors bacterial translocation[8-12]. Endotoxemia plays a critical role in the exacerbation of host and acquired immune responses, activation of cells to release cytokines, which can promote intestinal and liver tissue damage[13-16]. Furthermore, bacterial translocation (BT) is associated with severity of liver disease and provokes serious clinical events and complications[17].

THE INTESTINAL BARRIER STRUCTURAL AND FUNCTIONAL ELEMENTS

The intestinal tract represents the body's largest interface between the host and the external environment. The complexity of its function is obvious when thinking that the intestine has to serve simultaneously two distinct functions; the absorption and transport of necessary nutrients from the intestinal lumen into the circulation and the internal milieu in general and, on the other hand, the prevention of the penetration of harmful entities including microorganisms, luminal antigens and proinflammatory factors. The latter function is known as barrier function. Gut barrier function depends on both the immune barrier, composed of locally acting factors, such as the secretory IgA, intramucosal lymphocytes, Payer's nodules, mesenteric lymph nodes and of the systemic host defense, the latter represented mainly by the reticuloendothelial system, the biological barrier-made up of normal intestinal flora responsible for colonization resistance - the mechanical barrier as well, consisted of the closed-lining intestinal epithelial cells and by the capillary endothelial cells. All these components of gut barrier integrity can be majorly affected by liver cirrhosis[12,18,19].

The intestinal mechanical barrier in cirrhosis

The intestinal mucosal barrier consists of the mucus layer and intestinal epithelial cells. The epithelium prevents translocation of pathogens *via* transcellular and paracellular route[20]. The enterocytes are connected to each other by junctional complexes consisting of TJs, adherens junctions, desmosomes, and gap junctions forming a selective physical barrier that regulates paracellular transport[21,22]. The main transmembrane protein families in tight junctions are members of the occludin, claudins, and junctional adhesion molecules, which are linked to the actin cytoskeleton regulating paracellular movement of micromolecules, bacteria and macromolecules such as lipopolysaccharide[22-24]. TJs regulate transport *via* two distinct pathways: a charge selective, claudin-based pores that are 4 Å in radius for small ions and uncharged molecules, and a second one pathway, regardless of molecules charge and size[25,26]. Liver cirrhosis induces prominent changes in enterocytes' tight junction proteins, representing a cellular mechanism for intestinal barrier disruption and hyperpermeability[19,27]. In cholestatic liver injury, increased myosin light-chain kinase activation and diminished expression of occludin and zonula occludens-1 (ZO-1) have been reported in colonic epithelium with a concomitant increased intestinal permeability[4]. Reduced expression of duodenal occludin and Claudin-1 has been found in patients with cirrhosis compared to controls. Also, these alterations were more apparent in decompensated patients as compared to compensated ones. Negative regression was proved between occludin and claudin-1 expression, Child-Pugh score, the size of esophageal varices and serum endotoxin levels. These data support the view that there is a dynamic relationship between portal hypertension, bacterial translocation and TJs expression in intestinal epithelial cells[19]. In patients with nonalcoholic steatohepatitis and alcoholic decompensated cirrhosis, increased Claudin-2 was proved and could comprise a pivotal factor inducing intestinal barrier disruption. Conflicting are the findings about TJ proteins ZO, occludin and claudin-1 and the gap junction protein Connexin expression[28]. In cirrhosis, one of the main contributing factors to TJ alterations is the increased production of tumor necrosis factor-α (TNF-α) by monocytes in mesenteric lymph nodes[29,30]. TNF-α increases miR-122a expression in Caco-2 enterocytes and *in vivo* in a mouse model. miR-122a binds to the noncoding region three prime untranslated region of occludin mRNA and impacts on occludin mRNA downregulation and subsequent

occludin diminished expression, as well as upregulates claudin-2 and -8 expression but does not induct any alteration of claudins-1, -3, -5. Moreover, a linear relationship between TNF-α induced reduction of occludin and a higher inulin flux has been observed, indicating an increased Caco-2 permeability to inulin[31].

Histopathological changes of intestinal mucosa: Specific ultrastructural alterations of intestinal mucosa have been observed in cirrhotic patients that may be related to increased BT. In a case control study of cirrhotic patients using electron microscopy, dilated extracellular space between adjacent enterocytes, more prominent in the lower portion of the intestinal epithelial cells and reduced number of shorter and thicker microvilli were observed[32]. In experimental models of cirrhotic rats the intestinal mucosa was presented with atrophic, shorter, fractured villi and infiltration of inflammatory cells into the lamina propria and the muscular layer. The glandular epithelia resembled as irregular structures after the loss of their cylindrical shape. Excessive villi swell and loose structure of mucous membrane were correlated positively to endotoxemia[33].

Mucus: The mucus layer overlying the intestinal mucosa provides a first line defense mechanism against harmful antigens, and prevents bacteria and their byproducts from invading the microvillus environment. Mucus consists of glycoproteins secreted by goblet cells called mucins. Mucin (MUC) secretion is affected by transcription factors [nuclear factor-κB (NF-κB)], growth factors, lipopolysaccharide (LPS), microbes presence, inflammatory cytokines[34,35]. NF-κB is activated during gastrointestinal tract inflammation and binds to specific sites in the promoter of MUC2[36]. Chronic alcohol feeding increases the mucus content in the small intestine in rats. Furthermore, increased mucus thickness has been observed in the duodenum of alcoholic patients as a concomitant protective modification[37,38]. Increased MUC2 and MUC3 mRNA expression has been found in the ileum of rats with liver cirrhosis compared to those without cirrhosis[2]. Intestinal mucus modulates bacterial adherence to the intestinal mucosal surface and is associated with a loss of intestinal barrier function[39].

Intestinal oxidative stress: Oxidative stress is a mediator of intestinal mucosal barrier damage in patients with liver cirrhosis, affecting intestinal epithelial cell apoptosis and proliferation, and enhances BT and endotoxemia[12]. Portal hypertension results in intestinal mucosa hypoperfusion and hypoxia, which exacerbate oxidative damage in the gut mucosa by the increased xanthine oxidase activity and oxygen free radicals release[11]. Xanthine oxidase found in the liver and intestinal mucosa catalyzes the oxidation of hypoxanthine to xanthine, the conversion of xanthine to uric acid and is an important source of free radicals in the intestinal epithelium. Increased xanthine oxidase and decreased xanthine dehydrogenase activity have been observed in the intestinal mucosa and enterocyte mitochondria in the state of liver cirrhosis. Oxidative stress causes tissue damage at the subcellular level by lipid peroxidation affecting mitochondrial function. Reactive oxygen species break down the cellular membrane stability and induct cell death by lipid peroxidation in the cirrhotic rats[9,11]. Increased levels of malondialdehyde, a product of the lipid peroxidation, have been found in ileal and cecal mucosa in cirrhotic rats with ascites when compared to control rats, and in cirrhotic rats with BT compared to those without BT[8,40]. Experimental cirrhotic rats received pentoxifylline treatment, a regimen which exerts anti-inflammatory and antioxidant effects, appeared to have lower malondialdehyde levels in the cecal mucosa compared to placebo-treated ones. Pentoxifylline administration attenuates bacterial overgrowth, BT to cecal lymph nodes and impacts on elimination of spontaneous bacterial peritonitis[40]. Free radicals can also affect viscosity of the mucus in the gastric mucosa, enhance bacterial adherence ability to the epithelial cells and facilitate the translocation across the mucosa, resulting in complications such as spontaneous bacterial peritonitis (SBP)[10,41].

The intestinal immunological barrier in cirrhosis

Gut-associated lymphoid tissue alterations: The host innate immune system is the first line defense mechanism which is activated against bacteria and other toxins. The intestinal immune system consists of the gut-associated lymphoid tissue, which comprises four lymphoid compartments: Peyer's patches, lamina propria lymphocytes, including dendritic cells (DCs), intraepithelial lymphocytes and mesenteric lymph nodes, which are implicated in both the adaptive and innate immune defense mechanism[42]. The interaction between the host immune system and the microbiota inducts the activation of the intestinal immune system and the gut-associated lymphoid tissue that in turn modifies the microbiota environment[43]. DCs induce the development of Th1/Th17 T cells, regulatory T cells and promote TNF-α production[44]. Dendritic cells of the lamina propria induct tight junction alterations and sample microbes from the intestinal lumen[45]. An increased count of activated monocytes, dendritic cells and T lymphocytes in the intestinal mucosa and mesenteric lymph nodes (MLNs) coincided with specific alterations of cytokine expression in the intestinal mucosa as well as increased phagocytosis by intestinal dendritic cells in cirrhosis as a response to intestinal bacteria and other pathogens. Increased activated macrophages in the duodenal lamina propria, augmented intestinal permeability and altered intestinal tight junction protein expression have been demonstrated in patients with decompensated cirrhosis[28,31,46-48]. In response to BT, intestinal epithelial cells release chemokines, which exert chemoattractant effects and induce the recruitment of DCs to the mucosa as well as in MLNs[47]. IgA is one of the most important molecules in the regulation of intestinal homeostasis. Peyer's patches and isolated lymphoid follicles are

implicated in commensal-specific IgA production that aids to prevent the commensals from invading the gut mucosa[49]. Mice deficient in the toll-like receptors (TLR)-adapter molecule MyD88 on B cells lack commensal-specific immunoglobulin-response that results in impaired epithelial integrity and enables commensal bacteria to function as highly pathogenic organisms[50]. A pronounced reduction in $CD27^+$ memory B-cells count and functional capacity as well as a reduced ability to recruit T-cells, have been observed in cirrhotic patients. These B-cell defects may explain the susceptibility to bacterial infection. Also blockade of TLR4 and TLR9 signaling abrogates the activation of normal donor B-cells by cirrhotic plasma, suggesting a role for bacterial translocation in cirrhosis[51]. T cells are critical in host defense against the translocation of enteric bacteria since their depletion has been correlated with augmented BT and spreading of bacteria to extraintestinal sites and MLNs[37,52,53].

Antimicrobial peptides: Deficiency in antimicrobial peptides (AMPs) leads to disruption of the mucosal barrier, a shift in the bacterial composition, bacterial overgrowth and increase in BT. Antimicrobial peptides, also called host defense peptides, are part of the innate immune response and act as broad spectrum antibiotics killing Gram negative and Gram positive bacteria, viruses and fungi. AMPs include defensins, cathelcidins, lysozyme, resistin-like molecules and lectins. Defensins have a broad range of antimicrobial activity by binding to the microbial cell membrane and forming pore-like membrane defects. Human a-defensins that are expressed by neutrophils and Paneth cells located at the base of Lieberkuhn crypts, in response to bacteria and LPS exposure, regulate and maintain microbial balance in the intestinal lumen[54-56]. Reduced expression of Paneth cell defensins and diminished *in vitro* antibacterial activity of a-defensins against *Enterobacteriacea* have been observed in ascitic cirrhotic rats with BT to MLNs[57]. Regenerating islet derived proteins RegIII, produced by Paneth cells *via* activation of TLRs by pathogen-associated molecular patterns, bind to cell wall peptidoglycans of Gram-positive bacteria, and maintain a physical barrier between the epithelial cell surface and intestinal microbes[58,59]. Chronic alcohol intake has been shown to diminish RegIII expression in the small intestine of mice as well as in humans[3]. IgA antibodies released into the intestinal lumen, bind and aggregate bacteria, preventing mucosal adherence and colonization[60]. Reduced fecal IgA content as well as diminished secretion of mucosal IgA into the jejunum have been reported, suggesting a potential relationship between IgA, BT and development of infections in cirrhosis[37,61].

Cytokine alterations in cirrhosis and immune dysfunction: Endotoxemia as a result of intestinal barrier dysfunction, triggers the activation of the innate immune system and the release of proinflammatory cytokines[62]. The increased proinflammatory cytokine production (TNF-α, IFN, IL-6) and reduced anti-inflammatory cytokines (IL-10), in state of liver cirrhosis, by intestinal immune cells, affect the intestinal epithelial barrier integrity disrupting the epithelial tight Junctions and favour the increase of bacterial translocation[29,33,63,64]. Insulin-like growth factor Ⅰ therapy in cirrhotic rats has been found to promote portal pressure, bacterial translocation and endotoxemia reduction through diminished TNF-α expression[65].

The intestinal biological barrier in cirrhosis

Gut microbiota alterations: Intestinal bacterial overgrowth is common in cirrhosis and it has been shown to be particularly frequent in those with more severe liver disease and in those with a prior history of SBP and/or hepatic encephalopathy[66-71]. Reduced gastric acid secretion, intestinal dysmotility, lack of bile salts and reduced antimicrobial peptides killing capacity as well as portal hypertension have been recognized as contributory factors to IBO[3,72,73]. Changes in the gut microflora favor bacterial translocation and promote endotoxemia in patients with cirrhosis and experimental models of cirrhosis[67,74,75]. A direct relationship between the density and composition of cecal bacteria and the number of viable bacteria of this strain, present in MLNs, has been demonstrated in mouse models[76]. Intestinal bacterial overgrowth promotes the development of SBP by increasing bacterial translocation. Aerobic bacteria in cecal stool are increased in cirrhotic rats with bacterial translocation with or without spontaneous bacterial peritonitis compared to cirrhotic rats without bacterial translocation and SBP[72]. The impaired motility of the small intestine is a common feature in cirrhosis and may be a crucial factor in the pathophysiology of intestinal bacterial overgrowth, increased intestinal permeability and subsequent bacterial translocation[77]. The small intestinal transit is delayed in cirrhotic rats and the cecal aerobic bacteria count is higher compared to healthy controls[78].

CLINICAL IMPLICATIONS

Liver injury

Intestinal inflammation and bacterial translocation play a major role in the progression of liver fibrosis *via* TLR2, the receptor for products from Gram-positive bacteria such as peptidoglycan which in turn promotes a cascade of signals on monocytes in the lamina propria and tumor necrosis factor receptor type Ⅰ (TNFR Ⅰ) on intestinal epithelial cells. $TLR2^{-/-}$ mice have shown significantly less positive mesenteric lymph node cultures and lower endotoxin levels in the systematic circulation as a marker of bacterial translocation compared to wild type mice. TNFR $Ⅰ^{-/-}$ mice are protected from liver fibrosis by a decreased collagen α (I) gene expression and deposition of extracellular matrix proteins, suggesting that TNFR Ⅰ on intestinal epithelial cells enhances the paracellular leakage and favors bacterial translocation

and liver fibrogenesis[46]. LPS leads to host immune activation and enhances plasma sCD14 as a response. In patients with severe fibrosis higher plasma levels of sCD14 and more hepatic $CD14^+$ cells have been documented compared to patients with minimal fibrosis. LPS-mediated activation of both circulating monocytes and hepatic Kupffer cells induces liver fibrosis and progression to end-stage liver disease[79]. Seki *et al*[80] demonstrated that the intestinal bacterial microflora and a functional TLR4 are required for hepatic fibrogenesis. Hepatic stellate cells (HSCs) are the target through which TLR4 ligands such as lipopolysacharide promote fibrogenesis. In quiescent HSCs, TLR4 activation triggers chemokine secretion, induces chemotaxis of Kupffer cells, downregulates the transforming growth factor (TGF)-b, sensitizes HSCs to TGF-b - induced signals and allows unrestricted activation by Kupffer cells. LPS-induced HSCs sensitization to TGF-b leads to collagen production and deposition and seems to be mediated by a MyD88-NF-κB-dependent pathway[80].

Hepatocellular cancer

The majority of hepatocellular cancer (HCC) cases are generated in the state of chronic liver inflammation. Increased intestinal permeability, bacterial translocation and LPS accumulation activating the NF-κB pathway, suggest a hallmark of chronic liver disease and contribute to hepatic inflammation, proinflammatory cytokines TNF-α, IL-6 and IL-1 release, oxidative damage and fibrosis. The deterioration of normal equilibrium in the intestinal microbiota and NF-κB activation through upregulation of TNF-α exert promotional properties in HCC development[81]. Decreased hepatocarcinogenesis has been found in mice lacking IKK-b, a kinase required for NF-κB activation, in both hepatocytes and hematopoietic-derived Kupffer cells, suggesting that IKK-b orchestrates inflammatory crosstalk between hepatocytes and Kupffer cells and promotes liver cancer induction[82]. Infusion of LPS, which is an agonist of Toll-Like Receptor, increases hepatocarcinogenesis, tumor number and size in experimental animal model of mice intoxicated with DEN/CCl4. In advanced liver disease HCC development is mediated by TLR4-dependent secretion of growth factors such as epiregulin hepatomitogen by hepatic stellate cells, leading to EGFR and HER2 activation during the first stages of carcinogenesis, whereas it reduces hepatocyte apoptosis by NF-κB nuclear translocation[83-85]. TLR4 deficiency and antibiotic-induced gut sterilization decrease hepatic proliferation and fibrogenesis and could prevent HCC in patients with chronic liver injury, suggesting that the intestinal microbiota and TLR4 overexpression represent a possible molecular mechanism for the induction of HCC promotion[84]. These data suggest that disturbances of intestinal microflora, endotoxemia, and subsequent TLR4 mediated hepatic stellate cell activation might provide a dynamic interplay between endotoxemia, hepatic fibrosis and HCC promotion by increasing growth factors[83,85,86]. The hepatic expression of the glutathione S-transferase placental form, a marker for cellular alteration in the early stage of HCC development, has decreased in rats treated with probiotic MIYAIRI 588 compared to the choline deficient amino acids - diet-fed rats. The number and the size of the HCC lesion reduction in the MIYAIRI 588-treated rats have been correlated with endotoxemia elimination and increased ZO-1 and occludin expression, suggesting that bacterial translocation enhancement may constitute a promoting factor in hepatocarcinogenesis[87].

Hepatic encephalopathy

Intestinal dysbiosis and bacterial infections are precipitating factors for the induction of hepatic encephalopathy overt or subclinical. In previous studies cognitive impairment was recorded in 42% of cirrhotics without infection, in 79% of those with infection and without SIRS and in 90% of septic patients[88,89]. Altered flora, increased endotoxin levels, and excessive inflammation (IL-6, TNF-α, IL-2, and IL-13) have been found in cirrhotics with HE compared with those without hepatic encephalopathy (HE)[90]. Streptococcus salivarius is more prominent in cirrhotic patients with minimal hepatic encephalopathy (MHE) in comparison to those without HE, and is significantly associated with ammonia concentration[91]. Bacterial overgrowth with abundance of Gram-negative [*Escherichia coli* (*E. coli*)] and Gram-positive (*Staphylococcus* spp.) has been associated with cirrhosis complicated with MHE[92]. A higher incidence of previous hepatic encephalopathy episodes has been revealed in patients with TLR4 D299G and/or T399I polymorphisms, which are associated with intestinal barrier dysfunction, compared to wild-type patients (78% *vs* 20%)[93].

Gastrointestinal bleeding

Bacterial infection might increase the risk of variceal hemorrhage[94,95]. Cirrhotic patients with impaired intestinal permeability, high lipopolysaccharide binding protein and IL-6 levels represent a higher risk of variceal bleeding[96]. Bacterial infection is responsible for early rebleeding[95]. In a prospective study by Bernard *et al*[97], early rebleeding, defined as recurrence of bleeding within 7 d after admission, was observed in 43.5% of patients with bacterial infection compared to 9.8% in those without infection. Furthermore, the mean number of blood units transfused and the 4-wk mortality were significantly higher in patients with infection[97]. Bacterial infection was independently associated with failure to control bleeding in a previous study[98]. Patients with hepatocellular carcinoma and variceal bleeding tend to have a greater rebleeding rate due to a higher infection rate. Antibiotic prophylaxis can prevent infection and rebleeding, improving survival rate as well as decreasing the amount of blood transfused in patients with acute gastroesophageal variceal bleeding following endoscopic treatment[99,100]. A retrospective study suggested that administration of antibiotics prior to endoscopy or up to 8 h following endoscopy, if this is initially missed,

reduces rebleeding and improves 28-d survival[101,102].

Hepatopulmonary syndrome

Bacterial translocation and subsequent endotoxemia in cirrhotic rats may be a pathogenetic mechanism implicated in hepatopulmonary syndrome (HPS) progression. Endotoxin mediated stimulation of Kupffer cells *via* mitogen-activated protein kinase pathway upregulates TNF-α production and constitutes a key step in the induction of hepatopulmonary syndrome[103]. In cirrhotic rats endotoxemia, severity of liver disease and portal vein pressure are strongly correlated with the expression of eNOs, inducible nitric oxide synthase (iNOS), HO-1, histological changes in lung tissue, such as an increased number of dilated capillaries, infiltration of phagocytes and neutrophils and play a central role in the development of hepatopulmonary syndrome by inducing NO and CO[104]. In cirrhotic rats treated with norfloxacin, elimination of Gram-negative bacterial translocation, reduced count of pulmonary microvessels containing more than 10 macrophages, decreased expression and activity of lung iNOS have been observed, suggesting that bacterial translocation may be a major mechanism for the pathogenesis of HPS[105].

Hepatorenal syndrome

Hepatorenal syndrome is a specific type of renal failure that affects individuals suffering from liver cirrhosis[106]. Hepatorenal syndrome (HRS) is due to constriction of the blood vessels of the kidneys and dilation of the splachnic vessels which supplies the intestine[107]. Portal hypertension in cirrhosis has been associated with circulatory disturbances, arterial splanchnic vasodilatation and subsequent reduction in systemic vascular resistance, which results in reduced blood volume. Compensatory mechanisms such as vasoconstrictor systems and sodium retention in the kidneys are activated. However, increased cardiac output and hyperdynamic circulation, in advanced cirrhosis are insufficient to retain ideal intravascular effective volume resulting in hypoperfusion of kidneys[108,109]. The markedly decreased renal blood flow in decompensated cirrhosis, leads to hepatorenal syndrome that is frequently triggered from infections[110-112]. Patients with SBP without shock who exhibit high proinflammatory response are at high risk of developing kidney failure[111]. Renal failure occurs in approximately one third of patients with cirrhosis and bacterial infections and is irreversible or progressive in two-thirds of patients with treatment of infection only. The presence of a nosocomial infection, the absence of infection resolution with antibiotics and the peak count of neutrophil leukocytes in blood have been demonstrated as significant predictive factors of irreversibility of HRS[112-115]. Cirrhotic patients with culture-negative, non-neutrocytic ascites and bacterial DNA presence in ascitic fluid have a significantly higher TNF-α level in serum and ascitic fluid and a major risk of HRS compared to those without bacterial DNA, suggesting that bacterial translocation, subsequent inflammation and bacterial DNA presence are implicated in HRS induction[17]. Supportive to previous data are the results of Kalambokis *et al*[116] study, according to which intestinal decontamination with rifaximin therapy improves systemic circulation and renal function in patients with advanced alcoholic cirrhosis. Additionally, gut sterilization reduces CO and plasma renin activity, and inducts systemic vascular resistance increase. Rifaximin administration significantly improves the glomerular filtration rate and natriuresis while attenuates endotoxemia and reduces IL-6 and TNF-α production, suggesting that the prevention of infection in cirrhotic patients with renal failure seems to be a beneficial approach[116,117].

Infections

The intestinal permeability index (IPI) is increased in patients with advanced liver cirrhosis and active gastrointestinal hemorrhage, especially in those with proven or possible infections. IPI is an independent factor for the prediction of infection incidence in cirrhotic patients, suggesting that intestinal barrier dysfunction inducts bacterial translocation and affects the patient susceptibility to infections[118]. Patients with a bacterial infection suffer from a more severe liver disease with lower serum albumin and prolonged prothrombin time compared to cirrhotics without signs of infection[119]. Rimola *et al*[120] demonstrated that decompensated cirrhotics with a depressed reticuloendothelial system phagocytic activity have a higher risk of bacteremia affecting the survival rate.

Spontaneous bacterial peritonitis

Spontaneous bacterial peritonitis is a common complication of cirrhosis. Bacterial contamination of ascites fluid leading to SBP is caused by bacterial translocation. In cirrhotic rats identical bacterial species are cultivated in both mesenteric lymph nodes and ascitic fluid[121]. Among the patients with liver cirrhosis and culture-negative, non-neutrocytic ascites has been documented that the presence of ascitic bacterial DNA coincides with a higher relative risk of spontaneous bacterial peritonitis, suggesting a distinct association of SBP with impaired intestinal barrier function and increased bacterial translocation[17]. Patients with decompensated cirrhosis carrying Nucleotide-binding oligomerization domain containing 2 (NOD2) risk alleles (1007fs, G908R, R702W) which have been linked with impaired intestinal barrier or a history of prior SBP are at significant risk for development of spontaneous bacterial peritonitis and bacterascites[122,123]. It remains controversial whether proton-pump inhibitors use increases bacterial translocation and the risk of SBP[124-126]. On the other hand, treatment with b-blockers may prevent spontaneous bacterial peritonitis[127].

MORTALITY

Patients with liver cirrhosis and bacterial DNA in ascites as molecular evidence of intestinal bacterial

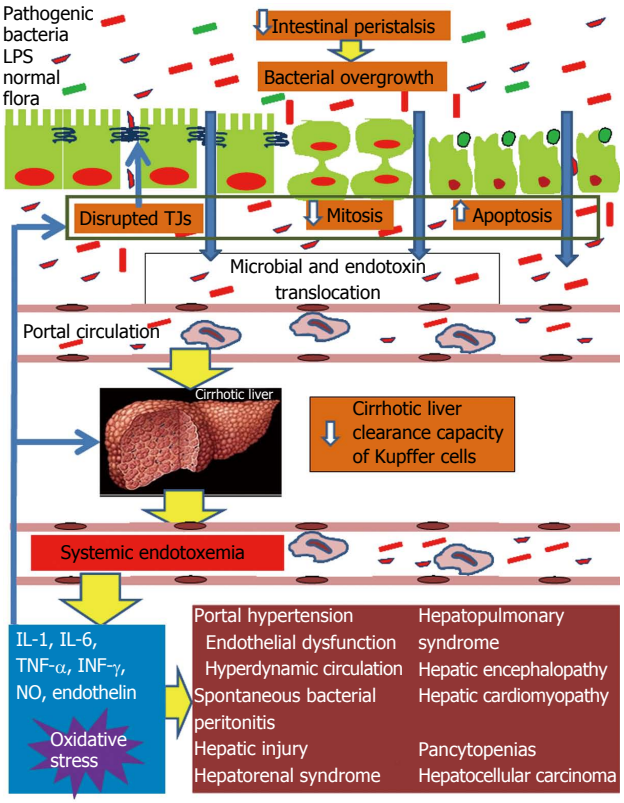

Figure 1 Pathophysiological overview of intestinal barrier dysfunction in liver cirrhosis and its interconnection with cirrhosis' complications from diverse organs. Liver cirrhosis delays intestinal motility thus changing the indigenous gut microecology and promoting intraluminal bacterial and endotoxin overgrowth. In parallel, the structural and functional integrity of the intestinal mucosa is disrupted leading to increased gut permeability. Important factors implicated in increased intestinal permeability are the disruption of the tight junctions structural complex and altered epithelial homeostasis, with decreased mitotic activity and increased apoptosis of enterocytes. Systemic cytokinemia and oxidative stress are pivotal promoters of these intestinal alterations. Increased gut permeability permits the escape of intraluminal bacteria and endotoxins initially into portal blood and subsequently, through a decreased clearance capacity of the cirrhotic liver, into systemic circulation. Systemic endotoxemia activates a systemic inflammatory response with release of interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha, interferon-γ, nitric oxide and endothelin-1, which can induce circulatory and remote organ dysfunction, partially through promotion of reactive oxygen species formation in the endothelium, lung, kidney, brain, heart and bone marrow. At the same time, the endotoxin-induced increased systemic levels of proinflammatory cytokines and oxidative stress aggravate intestinal and hepatic injury, further promoting bacterial translocation and endotoxemia, thus, maintaining the vicious cycle of gut barrier dysfunction, bacterial and endotoxin translocation, systemic release of proinflammatory cytokines and oxidative stress, complications of cirrhosis from diverse organs. TJ: Tight junction; LPS: Lipopolysaccharide.

translocation have an increased risk of death compared to those without bacterial DNA[17]. *NOD2* gene variants in patients with advanced liver cirrhosis linked to impaired mucosal barrier function may be genetic risk factors for death. NOD2 risk alleles and spontaneous bacterial peritonitis are independent predictive factors of death[122,123]. In a prospective study of fifty-three patients with cirrhosis, univariate Kaplan Meier analysis showed that Child-Pugh group, serum bilirubin, serum albumin, plasma endotoxin, and prothrombin time were associated with mortality[67].

CONCLUSION

In conclusion, intestinal barrier function is impaired in patients with cirrhosis and this derangement seems to be more pronounced in advanced cirrhosis. The disruption of mucosal barrier integrity is multifactorial, depends on a series of cellular and immune-mediated events, and affects the natural history of liver disease and patients' survival as illustrated in the Figure 1. Therefore, there is an open field for clinical investigations intending new customized treatment interventions at a molecular level and the modification of bacterial translocation events.

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P- Reviewer: Decorti G, Zoller M **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i17.2069

World J Hepatol 2015 August 18; 7(17): 2069-2079
ISSN 1948-5182 (online)

REVIEW

Prediction of liver cirrhosis, using diagnostic imaging tools

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Author contributions: All the authors contributed to this work.

Conflict-of-interest statement: We disclose that authors have no conflict of interest about this manuscript.

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Received: April 28, 2015
Peer-review started: May 6, 2015
First decision: June 25, 2015
Revised: July 15, 2015
Accepted: August 10, 2015
Article in press: August 11, 2015
Published online: August 18, 2015

Abstract

Early diagnosis of liver cirrhosis is important. Ultrasound-guided liver biopsy is the gold standard for diagnosis of liver cirrhosis. However, its invasiveness and sampling bias limit the applicability of the method. Basic imaging for the diagnosis of liver cirrhosis has developed over the last few decades, enabling early detection of morphological changes of the liver by ultrasonography (US), computed tomography, and magnetic resonance imaging (MRI). They are also accurate diagnostic methods for advanced liver cirrhosis, for which early diagnosis is difficult. There are a number of ways to compensate for this difficulty, including texture analysis to more closely identify the homogeneity of hepatic parenchyma, elastography to measure the stiffness and elasticity of the liver, and perfusion studies to determine the blood flow volume, transit time, and velocity. Amongst these methods, elastography using US and MRI was found to be slightly easier, faster, and able to provide an accurate diagnosis. Early diagnosis of liver cirrhosis using MRI or US elastography is therefore a realistic alternative, but further research is still needed.

Key words: Liver fibrosis; Ultrasonography; Computed tomography; Magnetic resonance imaging; Magnetic resonance elastography; Sonoelastography; Acoustic radiation force impulse imaging

Core tip: The development of new imaging modalities for liver cirrhosis has enabled early and accurate diagnosis of liver cirrhosis. Currently, elastography, used to measure the stiffness and elasticity of the liver, is more widely applied than texture. Ultrasound is simple imaging tool in diagnosing cirrhosis and can be added as several additional complementary technologies. The non-inferior diagnostic capability, non-invasiveness and relative cost-effectiveness of ultrasonography elastography may enable it to be one of the most useful techniques for diagnosis of liver cirrhosis.

Yeom SK, Lee CH, Cha SH, Park CM. Prediction of liver cirrhosis, using diagnostic imaging tools. *World J Hepatol* 2015; 7(17): 2069-2079 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2069.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2069

INTRODUCTION

Liver cirrhosis is the end stage of chronic liver disease. It is caused by diffuse fibrosis and regenerating nodules that result from recurrent necrosis of liver cell and degeneration. It is recognized as an irreversible form of parenchymal fibrosis. Liver cirrhosis reduces hepatic function and results in multiple complications induced by nodular regeneration and portal hypertension, including ascites, variceal bleeding, renal failure due to hepatorenal syndrome, hepatic encephalopathy, and spontaneous bacterial peritonitis. In addition, the incidence of hepatocellular carcinoma is sharply increased. Recently, early liver cirrhosis was shown to be improved by regression of collagen tissue[1]. Regression is usually associated with the improvement of clinical status, but can vary in the degree of improvement, depending on the reversibility of liver damage. Extensive scarring with parenchymal destruction is unlikely to regress. Therefore, early diagnosis of liver cirrhosis and quantification of the proportion of fibrosis in the liver are very important in the management of chronic liver disease. Prognosis and management of chronic liver diseases hinge strongly on the amount and progression of liver fibrosis[2,3].

There are a variety of causes of liver cirrhosis, with alcohol consumption, viruses, and fatty liver disease making up the majority of factors. These various etiologies induce chronic inflammation. Normal lobular architecture of the liver parenchyma is replaced by a parenchymal nodule surrounded by the fibrous tissue. Portal-central septa, connecting the portal vein and central vein, develop. As the inflammation persists, various form of fibrosis develops. The gross morphologic appearance of a cirrhotic liver is categorized by the size of the parenchymal nodules: micronodular, macronodular, or mixed. Micronodular cirrhosis is characterized by regenerative nodules of relatively uniform and small size. This pattern is seen in chronic alcoholic, hepatitis C, and biliary cirrhosis. In macronodular cirrhosis, the parenchymal nodules are larger, and more variable in size. Chronic hepatitis B is the most common cause of macronodular cirrhosis.

On the other hand, liver cirrhosis is classified according to the main location of fibrosis occurrence. A portal-based pattern usually results from hepatitis B and C, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, primary sclerosing cholangitis, recurrent pyogenic cholangitis, and hemochromatosis. Conversely, a centrizonal fibrosis pattern results from alcoholic and nonalcoholic steatohepatitis or chronic venous outflow obstruction.

There are differences in the grading and scoring of fibrosis by microscopic pathology according to the cirrhosis pattern. The METAVIR score (F0: no fibrosis, F1: portal fibrosis without bridging fibrosis, F2: portal fibrosis with few bridging fibrosis, F3: bridging fibrosis with architectural distortion, F4: cirrhosis) and the Ishak score (grades four categories of activity/necrosis, 0-4 or 0-6) are commonly used systems for grading or staging. The METAVIR score is simple, reproducible, and clinically validated, while the Ishak score is generally considered to be unnecessarily complex but preferred in many clinical trials[4].

Pathological confirmation of microscopic specimens obtained by ultrasound-guided needle biopsy is the reference standard for fibrosis staging. However, there are several well-known limitations, including sampling errors, subjective interpretation, semiquantitativeness, invasiveness, morbidity, and mortality of the procedure[5-7].

In clinical practice, the severity of liver cirrhosis is measured by multiple serologic biomarkers and many clinical scores and panels, such as the Child-Pugh score, model for end-stage liver disease score, FibroTest, HepaScore, FibroSpect, enhanced liver fibrosis score, and aspartate aminotransferase-to-platelet ratio index. However, these metrics also have many limitations, since the biomarkers are not liver-specific and measurement depends highly on their clearance and excretion[8,9].

Basic imaging diagnosis of liver cirrhosis has developed over the last few decades, enabling early detection of morphological changes of the liver using ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI). These methods are accurate for diagnosis of advanced liver cirrhosis. However, as morphological changes indicate advanced cirrhosis, there are limitations to early diagnosis of liver cirrhosis using imaging. To facilitate early diagnosis of liver cirrhosis, texture analysis and elastography to measure stiffness of the liver, and perfusion studies to determine the blood flow volume, transit time, and velocity were developed.

In this review, we highlight the many efforts made to improve diagnostic accuracy of imaging modalities in early liver cirrhosis.

IMAGING MODALITIES

The classical role of many imaging modalities in liver cirrhosis diagnosis is the detection of morphological changes in the liver. Cirrhotic liver shows nodular hepatic contour, changes in volume distribution, including an enlarged caudate lobe and left lobe lateral segment, atrophy of the right and left lobe medial segments, widening of the fissures and the porta hepatis, and regenerative nodules (Figure 1). Secondary findings related to portal hypertension may present, including varices, ascites, splenomegaly, fatty infiltration in the omentum and mesentery, edematous wall thickening of gastrointestinal tracts due to venous congestion, and intrahepatic arterioportal or arteriovenous shunts (Figure 2).

However, there are limitations to the diagnosis of early fibrosis, because these morphologic changes of the liver and related secondary findings represent advanced liver cirrhosis.

US

Ultrasound is a safe and relatively inexpensive imaging

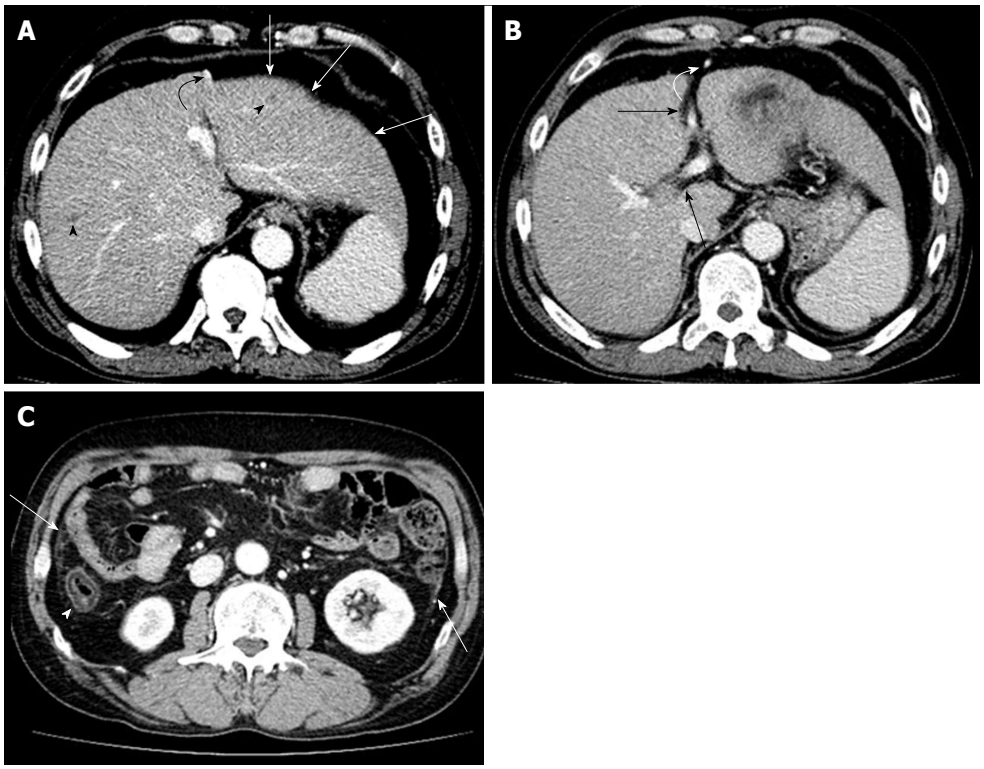

Figure 1 Contrast enhanced computed tomography portal phase images of the patient with liver cirrhosis due to chronic hepatitis B. A: Liver shows surface undulation (arrows). Two small low attenuated nodules are seen in both hepatic lobes suggesting regenerative nodules (arrow heads). Recanalized paraumbilical vein and paraesophageal varix are noted (curved arrow); B: Recanalization of paraumbilical vein (curved arrow) represents portal hypertension. Widening of hepatic fissure and porta hepatis is seen (black arrows); C: Ascending colon presents edematous wall thickening caused by congestion due to portal hypertension or hypoalbuminemic edema (curved arrow). Diffuse peritoneal thickening is also noted (arrows).

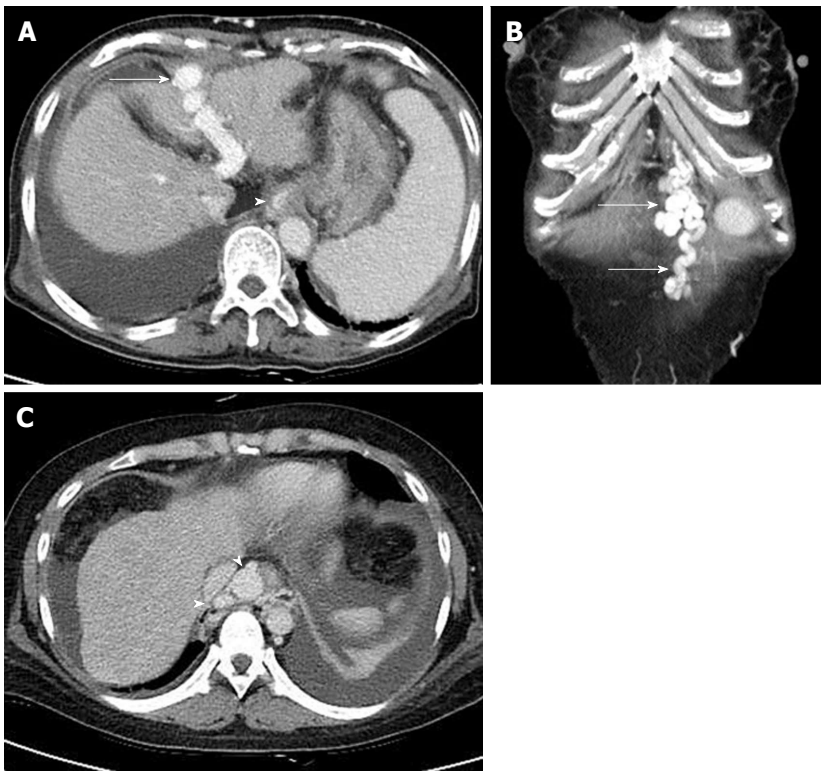

Figure 2 Image of liver cirrhosis caused by chronic hepatitis B. Contrast enhanced computed tomography portal phase images show multiple collateral vessels of portal vein. A: The image presents large intrahepatic portosystemic shunt through left portal vein and recanalized paraumbilical vein (arrow). Lower esophageal varix is seen (arrow head); B: Coronal image shows prominent paraumbilical veins (arrows); C: Axial image shows engorged paraesophageal varix (arrow heads) which usually supplied by left gastric vein and drained into azygos- or hemiazygos-vein.

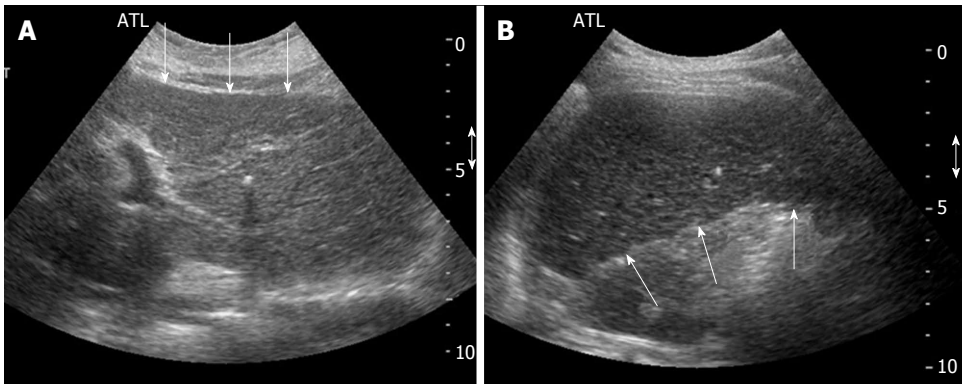

Figure 3 Transaxial scan. A: Transaxial epigastric scan shows the left lobe of the liver with surface irregularity (arrows), and coarse parenchyma echotexture; B: Subcostal transaxial scan shows the inferior margin of right hepatic lobe with irregular surface (arrows).

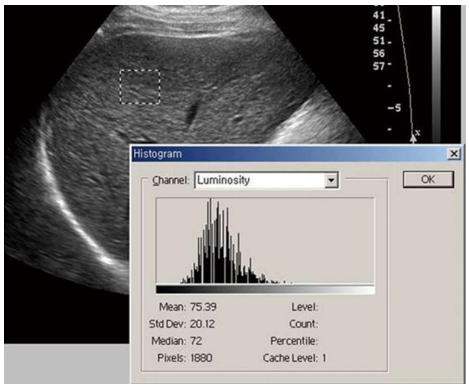

Figure 4 The region of interest of texture analysis is positioned in the right lobe of the liver, with an intercostals scan performed with gray scale ultrasonography. Chronic liver disease patient shows heterogeneous parenchymal echogenecity with high standard deviation value (Area: 1880 pixels, Mean: 75.39, SD: 20.12).

tool, allowing annual or biannual tests in chronic hepatitis patients. Initial findings of hepatic fibrosis by US are similar to simple hepatosteatosis[10]. Fibrosis of the hepatic parenchyma attenuates beam penetration, increases parenchymal echogenicity, and decreases vascular conspicuity.

Liver cirrhosis is characterized by changes in liver volume distribution, surface nodularity, accentuation of the fissure, heterogeneity, bright and coarsening of the hepatic architecture, cirrhotic nodules including regenerative and dysplastic nodules, and signs of portal hypertension. Studies showed an overall sensitivity to chronic liver disease of 65%-95%, with a positive predictive value of 98%[11-13]. The most indicative finding of liver cirrhosis was nodular surface, which was more sensitive on the undersurface of the liver than the superior surface (86% *vs* 53%) (Figure 3). It was also more sensitive in a high frequency probe[11-13]. Although any single US feature had limited sensitivity or specificity in detecting cirrhosis, improvements could be achieved by combining two or three parameters.

US imaging can provide early detection of morphological changes of the liver, but such changes represent advanced cirrhosis. Furthermore, ultrasound imaging is subjective and difficult to quantify, as inter- and intra-observer variability is a significant problem. There have been many efforts to objectively quantify the coarseness of hepatic parenchymal echogenicity. An initial study performed a simple quantification of parenchymal echogenicity and compared the standard deviation between chronic liver disease and normal liver (Figure 4)[14-16]. The coarseness of hepatic parenchyma decreased beam penetration, while the attenuation of echogenicity according to depth increased proportionally to fibrosis. Methods that were more delicate were also introduced. Measurement of differences in echogenicity between neighboring pixels can be pathologically correlated to chronic liver disease[17]. Texture analysis can improve diagnostic accuracy of grayscale US images. However, there are several limitations to the widespread use of these techniques, including dedicate post-processing programs, inter-observer variability, and sampling bias. The success of this approach also depends strongly on an expert to establish the regions of interest[18].

In cirrhosis, Doppler waves of the hepatic vein show spectral broadening and hepatic vein narrowing. Phasic oscillations in hepatic venous flow are dampened. Normal phasicity of the hepatic vein represents a pressure change in the right atrium through the cardiac cycle. However, phasicity of the hepatic vein is reduced in liver cirrhosis, a result of decreased hepatic compliance and venous segments narrowed by adjacent regenerative nodules[19]. The portal vein is initially dilated over 1.4 cm in diameter, but the emergence of the bypass collateral vessels changes hepatofugal blood flow and decreases the portal vein diameter to less than 1 cm. The hepatic artery has a high resistive index, but the development of a large arteriovenous shunt or arterioportal shunt leads to lower resistance[20-22].

Development of contrast materials using microbubbles can help measure the blood transit time of the liver. Hepatic arterial/vein transit time decreases with fibrosis progression. It is known that intrahepatic arterioportal or arteriovenous shunt and arterializations of the cirrhotic liver leads to short blood transit times[23,24]. However, these studies showed no significant

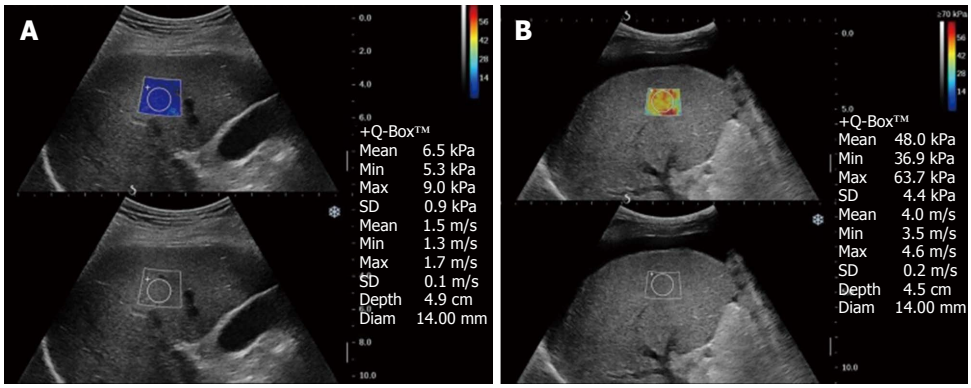

Figure 5 The region of interest of supersonic shear wave imaging is positioned in the right lobe of the liver, with an intercostals scan. On the right of the display there is shear wave velocity (expressed in kPa and m/s). The display show a real-time color mapping of the elasticity encoded pixel by pixel in an image superimposed on the standard B-mode. In panel (A) subject with normal shear wave speed value. In panel (B) patient with shear wave speed value compatible with liver cirrhosis. The display also shows large amount of ascites.

correlation between the severity of hepatic fibrosis and hemodynamic coefficients including hepatic vein transit time, hepatic artery transit time and intrahepatic transit time. The reason is that blood transit time is influenced by not only arterioportal or arteriovenous shunt, but also various extrahepatic and intrahepatic factors such as cardiac output and the degree of first-pass phagocytosis of contrast agent by Kupffer cells[23].

US elastography is now widely recognized as a reliable method to assess liver fibrosis. The principle of elastography is the shearing of the examined tissue, which induces a smaller strain in hard tissues than in soft ones. There are several commercial types of US elastography currently in use: transient elastography (TE), acoustic radiation force impulse imaging (ARFI), Supersonic shear wave imaging (SSI), and real-time tissue elastography.

TE is performed with the Fibroscan™ (Echosens, Paris, France) which comprises of an ultrasound transducer probe located on the axis of a vibrator. The vibrator makes a vibration, which leads to an elastic shear wave propagating to the liver. The shear wave velocity (expressed in kiloPascals-kPa) is directly related to the stiffness of the tissue[25]. At present, TE is the most widely used method for the liver fibrosis assessment. TE has been validated in various chronic liver diseases including chronic hepatitis B and C, nonalcoholic fatty liver disease[26-29]. However, it has several limitations; the rate of unreliable measurements is reached up to 20% and the rate of reliable measurements decreased in obese patients and it cannot be performed in patients with ascites[30].

ARFI technique is directly integrated on a standard US machine and shear wave is localized, allowing selection of the region of interest (ROI) by the operator on a real time US image. The ultrasound probe automatically produces an acoustic "push" pulse, generating shear-waves that propagate into the tissue. Transmission of a longitudinal acoustic pulse leads to tissue displacement, resulting in shear-wave propagation away from the region of excitation. Shear-wave velocity (given in m/s) is measured within a defined ROI using US tracking beams laterally adjacent to the single push beam[31]. Propagation speed increases with fibrosis severity. Results were similar to those with transient elastography[29,32].

In contrast to TE and ARFI using a single shear wave emitted temporarily at a single frequency for each measurement, the ultrasound transducer of SSI technique (Aixplorer, Supersonic Imagine, Aix-en-Provence, France) emits a multiple pulse wave beams at increasing depths allowing the evaluation of the velocity of several shear wave fronts over a wide frequency range at the same time. By generating a real-time color mapping of the elasticity encoded pixel by pixel in an image superimposed on the standard B-mode, SSI allows to show the viscoelastic properties in all areas of an ROI with a color look-up table (Figure 5). This is expected to overcome the limitations of transient elastography, where liver stiffness cannot be measured accurately in patients with severe obesity, and ascites. Some articles have shown growing evidence for the accuracy of US elastography[33-37] (Table 1). Although the low reproducibility of measurements derived from operator-dependent performance remains a significant limitation of US elastography, this technique is a useful diagnostic tool for hepatic fibrosis and further validation is warranted.

CT

CT is the most sensitive diagnostic tool for evaluating hepatic morphological changes[38]. CT readily shows alterations in hepatic morphology and extra-hepatic manifestations related to portal hypertension. With liver cirrhosis progression, the nodularity of the liver surface and generalized heterogeneity of the hepatic parenchyma are visible. The porta hepatis and interlobar fissure frequently appear wider due to shrinkage of the right lobe and the medial segment of the left lobe with concomitant enlargement of the caudate lobe and the lateral segment of the left lobe. Changes in size and volume distribution are easily visible in a CT

Table 1 Diagnostic performance of ultrasonography elastography for hepatic fibrosis

Ref.	Year	Study method	Imaging instrument	Etiologies	No. of patients	Sensitivity (%)	Specificity (%)	Cut-offs	AUROC	Fibrosis score
Tada *et al*[33]	2015	Prospective	SSI	HCV	55	88.9	91.9	8.8 kPa	0.94	F2-3 (F4, excluded)
Samir *et al*[34]	2015	Prospective	SSI	Chronic viral and nonviralhepatopathies	136	91.4	52.5	7.29 kPa	0.84	≥ F2
Deffieux *et al*[63]	2015	Prospective	SSI	Chronic viral and nonviralhepatopathies	120	77	79	8.9 kPa	0.81	≥ F2
Yoon *et al*[35]	2014	Prospective	SSI	Chronic viral and nonviralhepatopathies	94	78.8	75.6	6.65 kPa	0.852	≥ F2
Tutar *et al*[36]	2014	Prospective	SSI	Children, viral and nonviralhepatopathies	76	97.8	96	10.4 kPa	0.96	≥ F2
Jeong *et al*[64]	2014	Prospective	SSI	Chronic viral and nonviralhepatopathies	70	78.2	93.3	8.6 kPa	0.915	≥ F2
Cassinotto *et al*[37]	2014	Prospective	SSI	Chronic viral and nonviralhepatopathies	336	83	82	8 kPa	0.89	≥ F2
			ARFI	Chronic viral and nonviralhepatopathies	341	72	81	1.38 m/s	0.81	≥ F2
			TE	Chronic viral and nonviralhepatopathies	337	76	81	8.5 kPa	0.83	≥ F2
Yap *et al*[31]	2013	Prospective	ARFI	Chronic viral and nonviralhepatopathies	70	68	66	1.55 m/s	0.85	≥ F2
Bota *et al*[32]	2013	Meta-analysis	ARFI	Chronic viral and nonviralhepatopathies	1163	74	83	1.30 m/s	0.85	≥ F2
			TE	Chronic viral and nonviralhepatopathies	1163	78	84	N/A	0.87	≥ F2
Ferraioli *et al*[65]	2012	Prospective	SSI	HCV	121	90	87.5	7.1 kPa	0.92	≥ F2
Chon *et al*[29]	2012	Meta-analysis	TE	HBV	2772	74.3	78.3	7.9 kPa	0.859	≥ F2
Friedrich-Rust *et al*[26]	2008	Meta-analysis	TE	Chronic viral and nonviralhepatopathies	8433	N/A	N/A	7.65 kPa	0.84	≥ F2

AUROC: Area under receiver operating characteristic; SSI: Supersonic shear wave imaging; HCV: Hepatitis C virus; ARFI: Acoustic radiation force impulse imaging; TE: Transient elastography (FibroScan™); HBV: Hepatitis B virus; N/A: Not applicable.

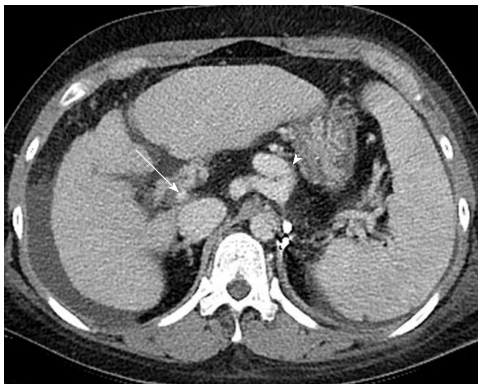

Figure 6 Image of liver cirrhosis caused by chronic hepatitis B. Contrast enhanced computed tomography portal phase image shows the liver with irregular surface and heterogeneous enhancement of parenchyma with reticular pattern suggesting confluent fibrosis. The image shows decreased diameter of portal vein (arrow) due to large collateral vessels (arrow head) and also shows large amount of ascites.

scan. In early stages, the liver may appear normal. The limited spatial resolution of CT and MRI allow detection of only a relatively thick fibrous septum. Thick fibrous septa and confluent hepatic fibrosis showed low attenuation in non-enhanced CT. The boundary between fibrosis and normal parenchyma was more ambiguous in a contrast-enhanced scan (Figure 6). Therefore, it is difficult to perform texture analysis using CT. Considering the fact that the CT contrast agent is an extracellular space contrast agent, texture analysis is a method of measuring the decrease of the extracellular space fraction. When liver cirrhosis progress is enforced experimentally, there is a high correlation with the fibrosis grade, though this has not been proven clinically[39].

Perfusion imaging in liver fibrosis is based on the occurrence of substantial microcirculatory changes in this disease. These changes are caused by capillarization of the sinusoids, collagen deposits in the extracellular space of Disse, and contraction of activated stellate cells[40]. Quantification of hepatic perfusion by dynamic CT has allowed separate evaluations of arterial and portal perfusion of the liver[41,42]. Perfusion CT can be used to detect microcirculatory changes that occur in cirrhosis and help to differentiate low-grade fibrosis[43]. Perfusion CT had several limitations. It suffered from the classic CT limitations: radiation, the use of iodinated contrast agents and limited scan coverage range[43]. However, new technological developments have reduced the scanning time and increased the detector size, enabling a reduction in the dose of radiation and expanding the scanning range.

MRI

MRI has several advantages over other imaging tech-

Table 2 Diagnostic performance of magnetic resonance elastography for hepatic fibrosis

Ref.	Year	Study method	Imaging instrument	Etiologies	No. of patients	Sensitivity (%)	Specificity (%)	Cut-offs	AUROC	Fibrosis score
Singh *et al*[66]	2015	Meta-analysis	1.5 Tesla, variable	Chronic viral and nonviralhepatopathies	697	79	81	3.66 kPa	0.88	⩾ F2
Venkatesh *et al*[67]	2015	Retrospective	1.5 Tesla, (GE, Signa)	Chronic viral and nonviralhepatopathies	62	100	96.5	3.37 kPa	0.99	⩾ F2
Yoon *et al*[56]	2014	Prospective	1.5 Tesla (GE, SignaHDx)	Chronic viral and nonviralhepatopathies	94	78.8	75.6	6.65 kPa	0.852	⩾ F2
Venkatesh *et al*[68]	2014	Prospective	1.5 Tesla (GE, Signa)	HBV	63	97.4	100	3.2 kPa	0.99	⩾ F2
Shi *et al*[69]	2014	Prospective	3.0 Tesla (GE, Signa Excite HD)	HBV	113	95	94.5	4.07 kPa	0.986	⩾ F2
Loomba *et al*[70]	2014	Prospective	3.0 Tesla (GE, Signa Excite HD)	Nonalcoholic fatty liver disease	117	86	91	3.63 kPa	0.924	⩾ F3
Bohte *et al*[71]	2014	Prospective	3.0 Tesla (Philips, Intera)	HBV, HCV	103	62	96	2.18 kPa	0.928	⩾ F2
Kim *et al*[72]	2013	Retrospective	1.5 Tesla (GE, Signa)	Nonalcoholic fatty liver disease	142	85	92.9	4.15 kPa	0.954	⩾ F3
Wang *et al*[73]	2012	Meta-analysis	1.5 Tesla, variable	Chronic viral and nonviralhepatopathies	972	94	95	N/A	0.98	⩾ F2
Rustogi *et al*[54]	2012	Retrospective	1.5 Tesla (Siemens, Magnetom)	Chronic viral and nonviralhepatopathies	72	85.4	88.4	5.9 kPa	N/A	⩾ F3
Kim *et al*[74]	2011	Prospective	1.5 Tesla GE, SignaHDx)	Chronic viral and nonviralhepatopathies	55	89.7	87.1	3.05 kPa	N/A	⩾ F2

AUROC: Area under receiver operating characteristic; HBV: Hepatitis B virus; HCV: Hepatitis C virus; N/A: Not applicable.

niques, including high-resolution images with excellent contrast against other soft tissue lesions and a number of different techniques facilitating the diagnostic evaluation of organ morphology, physiology, and function. As it is dependent on the detection of alterations in hepatic morphology, conventional MRI is limited to the diagnosis of earlier stages of liver fibrosis and is not suitable for disease staging[44].

Calculation of the apparent diffusion coefficient (ADC) with diffusion-weighted imaging (DWI) using MRI can facilitate the assessment of liver fibrosis[45]. One recent study showed that ADC values decrease with increasing stage of liver fibrosis from F0 to F4. However, no significant differences in ADC values were detected between the early stages of fibrosis[46,47].

Intravoxel incoherent motion (IVIM)-DWI was developed to quantitatively assess the microscopic translational motions of both intracellular and extracellular water molecules occurring in each voxel in MRI. Using IVIM imaging, several factors, such as pure molecular diffusion and microcirculation or blood perfusion, can be distinguished with multiple *b* values[48]. One study demonstrated that both ADC and perfusion-related diffusion (D*) were significantly reduced in the cirrhotic liver group compared with those in the healthy liver group, while there was no significant difference between pure molecular-based diffusion (D) and perfusion fraction (f) measurements in the healthy liver and cirrhotic liver groups[49]. ADC and D* reduction in cirrhosis represents reduced perfusion in cirrhotic liver. Another study showed the feasibility of IVIM parameters in differentiating early stages of fibrosis[50].

Magnetic resonance elastography (MRE) is an emerging technique that noninvasively quantifies liver stiffness by analyzing the propagation of mechanical waves through liver tissue. It is based on the concept that the stiffness of the hepatic parenchyma is increased as fibrosis advances. One study showed that MRE has a high sensitivity and specificity in detecting liver fibrosis, with a predicted sensitivity and specificity of 91% and 97% for liver fibrosis ⩾ stage F2, respectively; 92% and 95% for liver fibrosis ⩾ stage F3, respectively; and 95% and 87% for liver fibrosis ⩾ stage F4, respectively[51]. Another study showed a sensitivity and specificity of 98% and 99% for all grades of liver fibrosis with a cut-off value of liver stiffness of 2.93 kPa[52]. This study also showed that MRE could discriminate patients with moderate and severe fibrosis (grades 2-4) from those with mild fibrosis (sensitivity, 86%; specificity, 85%). Several studies show that MRE is more reliable for staging hepatic fibrosis compared to DWI and conventional MRI, with a powerful combination of sensitivity, specificity, likelihood ratios, diagnostic odds ratio, and area under the summary receiver operating characteristic curve values[51,53-55] (Table 2). MRE can be easily added to standard abdominal MRI protocols, promising value added in staging liver cirrhosis. One study showed that MRE and SSI results moderately correlated with liver cirrhosis values, though MRE measurements tended to be more reliable than US elastography[56].

MRE has many advantages: (1) it can exam the whole liver, with a lower sampling error than with a biopsy or other imaging modalities; (2) good diagnostic accuracy; and (3) the results are not influenced by hepatic steatosis, obesity, and ascites. However, some clinical limitations include: (1) misinterpretation of results due to a high iron overload in the liver, causing signal-

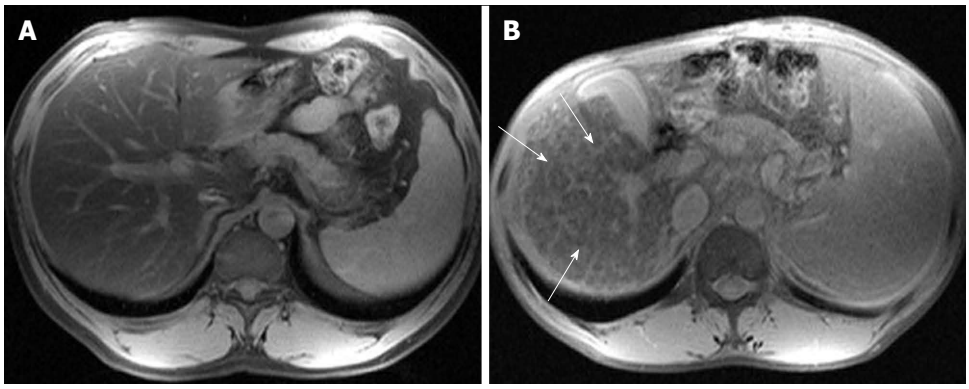

Figure 7 Double contrast enhanced protocol magnetic resonance images. Fat saturated T2-weighted magnetic resonance images of 15-min delay after injection of superparamagnetic iron oxides and gadolinium chelates. A: The image of normal patient shows homogenous low signal intensity of hepatic parenchyma; B: The image of patient with liver cirrhosis shows hyperintense reticulations (arrows), represent septal fibrosis, in cirrhotic liver.

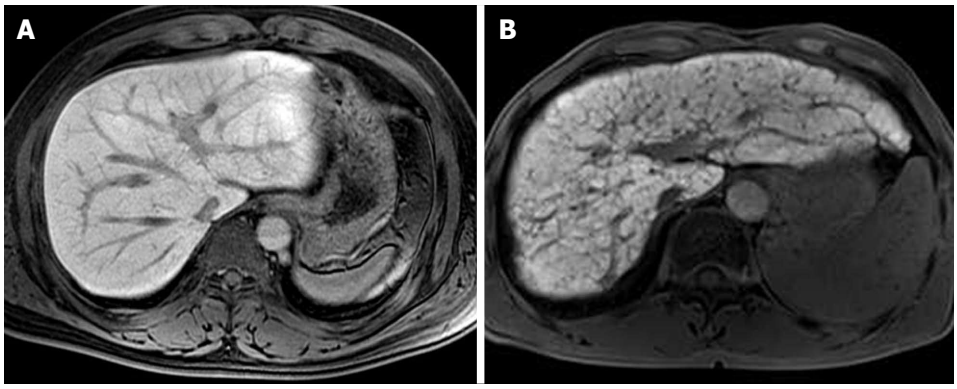

Figure 8 Fat saturated T1-weighted magnetic resonance images of 20-min delay after injection of gadoxetate disodium. A: The image of healthy patient shows homogenous high signal intensity of hepatic parenchyma; B: The image of patient with liver cirrhosis shows heterogeneity of hepatic parenchyma enhancement with hypointense reticulations representing septal fibrosis, and decreased enhancement degree as compared with the image (A).

to-noise limitations; (2) a longer examination time than SSI; (3) the need for dedicated installation equipment; and (4) a lack of comparable studies between 1.5 Tesla and 3.0 Tesla MRI machines and among other company products (Table 2). Therefore, an absolute cut-off value for diagnosis and grading of hepatic fibrosis has not been established. More research is needed.

Texture analysis of liver parenchyma to diagnose liver cirrhosis has been performed using contrast media. After injection of superparamagnetic iron oxides (SPIOs) or gadolinium chelates, hyperintense reticulations, which are postulated to represent septal fibrosis, can be observed in cirrhotic liver. It is known that SPIOs accumulate within liver reticuloendothelial cells after intravenous infusion, causing T2* shortening and reducing liver signal intensity. In cirrhotic liver, SPIOs accumulate and cause T2* shortening of normal liver parenchyma. Fibrosis appears in a hyperintense reticular pattern on T2 or T2* images. In addition, delayed T1 shortening and delayed enhancement of the hepatic fibrosis signal intensity by gadolinium chelates is expected (Figure 7). A double-contrast material-enhanced MRI protocol with sequential administration of SPIOs and gadolinium chelates was superior to a single-contrast material-enhanced MRI protocol for liver fibrosis diagnosis[57]. However, these protocols are no longer popular.

Gadoxetate disodium is a liver-specific MRI contrast agent with combined perfusion and hepatocyte-selective properties. Hepatocyte-phase gadoxetate disodium-enhanced MRI can be used to measure hepatocyte function[58-60]. One study shows that the contrast enhancement index (CEI = signal intensity post-enhancement/signal intensity pre-enhancement) in gadoxetate disodium-enhanced MRI more accurately correlated with hepatic fibrosis staging than ADC values (CEI: r = -0.79, ADC: r = -0.43)[61]. Another study shows that heterogeneity of hepatic parenchyma enhancement on Hepatocyte-phase gadoxetate disodium-enhanced MRI is correlated with the degree of liver parenchyma fibrosis using parameter of corrected coefficient of variation [cCV = (SDliver - SDair)/SIliver × 100] (Figure 8)[62].

CONCLUSION

The development of new imaging modalities for diagnosing of liver cirrhosis has led to the detection and measurement of subtle changes. This has enabled early and accurate diagnosis of liver cirrhosis. Currently, elastography, used to measure the stiffness and elasticity of

the liver, is more widely applied than texture analysis in diagnosis of liver cirrhosis. Results strongly correlate with hepatic fibrosis, without the need for a post-operation procedure. Although MRE has more accurate tendency, US is simple imaging tool in diagnosing cirrhosis and can be added as several additional complementary technologies. The non-inferior diagnostic capability, non-invasiveness and relative cost-effectiveness of US elastography may enable it to be one of the most useful techniques for diagnosis of liver cirrhosis.

We expect standardization of elastography techniques so that quantitative parameters obtained by clinical systems from different vendors may give similar results in the future.

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P- Reviewer: Song JS **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i17.2080

World J Hepatol 2015 August 18; 7(17): 2080-2090
ISSN 1948-5182 (online)

REVIEW

Immunology of hepatocellular carcinoma

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Author contributions: Arora SK conceived, read, and edited the manuscript; Chawla YK provided the clinical information and read the manuscript; Sachdeva M collected the literature, and compiled and wrote the manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest related to the manuscript.

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Received: March 31, 2015
Peer-review started: April 1, 2015
First decision: April 23, 2015
Revised: May 28, 2015
Accepted: July 18, 2015
Article in press: July 22, 2015
Published online: August 18, 2015

Abstract

Hepatocellular carcinoma (HCC) is primarily a malignancy of the liver, advancing from a damaged, cirrhotic liver to HCC. Globally, HCC is the sixth most prevalent cancer and the third-most prevalent reason for neoplastic disease-related deaths. A diverse array of infiltrating immunocytes regulates the development and progression of HCC, as is the case in many other cancers. An understanding of the various immune components during HCC becomes necessary so that novel therapeutic strategies can be designed to combat the disease. A dysregulated immune system (including changes in the number and/or function of immune cells, cytokine levels, and the expression of inhibitory receptors or their ligands) plays a key role in the development of HCC. Alterations in either the innate or adaptive arm of the immune system and cross-talk between them make the immune system tolerant to tumors, leading to disease progression. In this review, we have discussed the status and roles of various immune effector cells (*e.g.*, dendritic cells, natural killer cells, macrophages, and T cells), their cytokine profile, and the chemokine-receptor axis in promoting or impeding HCC.

Key words: Hepatocellular carcinoma; Immune cells; Immune-dysregulation; Adaptive immunity; Innate immunity

Core tip: Hepatocellular carcinoma (HCC) is a heterogeneous disease caused by multiple factors, and has its immunopathogenesis complicated by the paradoxical role of various immune cells. This review provides a comprehensive insight into the immunological mechanisms that control hepatocarcinogenesis. A better and fuller understanding of the precise function of each cellular subset may open new avenues for the treatment of HCC.

Sachdeva M, Chawla YK, Arora SK. Immunology of hepatocellular carcinoma. *World J Hepatol* 2015; 7(17): 2080-2090 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2080.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2080

INTRODUCTION

Hepatocellular carcinoma (HCC) is a cancer that originates in the liver, and is thus different from metastatic liver cancer that hails from other organs and culminates in the liver. Worldwide, HCC is the sixth most prevalent cancer, as well as being the third most common cause of mortality and poor-prognosis malignancy due to recurrence after surgery and metastasis[1]. It accounts for approximately 70%-80% of all primary liver cancer cases[2]. HCC is most prevalent in Asian nations like China and Japan, where it has a high mortality rate within weeks or months after detection. The disease is generally diagnosed at a late stage, which significantly brings down the survival rate to less than 14% within a span of five years[3]. The available treatment options are not 100% successful and the estimated recurrence rates are around 50% over a span of 3 years post-surgery and with a survival rate of only 30%-40% at five years post-surgery[4].

The major risk factors for chronic liver disease and subsequent HCC include prior infection with viruses like hepatitis B and hepatitis C[5]. Studies in mouse models have indicated the major role of local intra-hepatic chronic inflammation in promoting hepatocarcinogenesis in animals with non-alcoholic steatohepatitis (NASH)[6]. Accumulating data in humans also indicate an increasing role for NASH as a risk factor for HCC development[7]. In addition, other emerging risk factors are: obesity (especially visceral adiposity leading to non-alcoholic fatty liver disease), alcohol consumption, tobacco use, consumption of foodstuffs contaminated with aflatoxin B1, diabetes, overuse of oral contraceptive pills, and iron overload[4].

Factors promoting tumor antigen tolerance, such as decreased recognition of malignant cells, suppression of immunity, and chronic inflammation (either mediated by virus[8] or immune dysregulation), all lead to carcinogenesis[9]. Recent studies have provided evidence that a dysregulated immune system, including changes in the number or function of immune cells, cytokine levels, and expression of inhibitory receptors or their ligands significantly contribute to the development of HCC[10,11]. Alterations in the function or expression of immune components shift the immune response towards tumor tolerance, resulting in its progression. Tumor-related immune cells, such as cytotoxic T cells, $CD4^+$ T cells, regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells, and the cross-talk between these have all been reported to be involved in the development of HCC (Figure 1). In this review, we have discussed the immunology of HCC in terms of the status of various immune effector cells.

INNATE IMMUNE SYSTEM

Dendritic cells

Efficient recognition, processing, and presentation of tumor antigens by dendritic cells (DCs) are prerequisites for an effective immune response against tumors. Failed HCC-associated antigen presentation by DCs might not only be due to a decreased expression of human leukocyte antigen (HLA) class- Ⅰ molecules[11], as maturation defects like reduced endocytosis, allostimulation, and interleukin 12 (IL-12) secretion can lead to a weak T cell immune response[12]. Even in the presence of strong maturation stimuli like high levels of inflammatory cytokines, DCs remain refractory to these stimulatory signals. Studies have previously shown that there is a numerical and functional defect in the peripheral DCs in HCC patients with hepatitis B and C virus infections, although it is unclear whether this defect is a cause or an effect[13,14]. On the other hand, there have been reports that have shown the frequency of activated $CD83^+$ DCs in the peripheral circulation of HCC patients was comparable to patients with liver cirrhosis and normal healthy controls[15]. However, when compared to peripheral blood, activated DCs were present at a much lower frequency in the liver tissues of the other study groups. Additionally, the activated DCs in HCC patients were not able to infiltrate the cancer nodules, resulting in impaired recruitment of tumor-specific lymphocytes to tumor areas.

Recently, a new regulatory subset of DCs called $CD14^+$ cytotoxic T-lymphocyte-associated protein (CTLA)-4^+ DCs, which expresses inhibitory molecule-like CTLA-4 and programmed death receptor (PD)-1, were observed in the peripheral blood lymphocytes and tumor masses of HCC patients[16]. High levels of anti-inflammatory cytokine, IL-10, and indoleamine 2, 3-dioxygenase secreted by these cells' post-stimulation suppressed the $CD4^+$ T-cell immune response, thereby assisting tumor progression and immune escape.

Macrophages and myeloid-derived suppressor cells

Tumor-associated macrophages (TAMs) represent the main inflammatory cells associated with cancer-related inflammation[17]. While in infiltrating tumors, TAMs differentiate towards an M2 phenotype characterized by the expression of immunomodulatory cytokines [*e.g.*, IL-10 and transforming growth factor (TGF)-β] and poor antigen presentation capacity. TAMs also express chemokines like CCL17, CCL22, and CCL24, along with arginase and low levels of proinflammatory cytokines and reactive oxygen species[18]. In HCC, the cytokines IL-6 and TGF-β (in particular) favor tumor growth, tumor necrosis factor (TNF)-α and IL-6 are involved in invasion and metastasis, and TGF-β, in concert with IL-10, has been shown to promote the suppression of anti-tumor immune response[19]. This alternative phenotype of macrophages further participates in the activation of a T helper type 2 (Th2) immune response, thereby promoting the recruitment and development of Tregs. Chronic inflammation was reported primarily to be coupled with a higher prevailing level of macrophage colony stimulating factor and a higher infiltration of macrophages, which were reportedly associated with HCC progression and intrahepatic metastasis, thereby

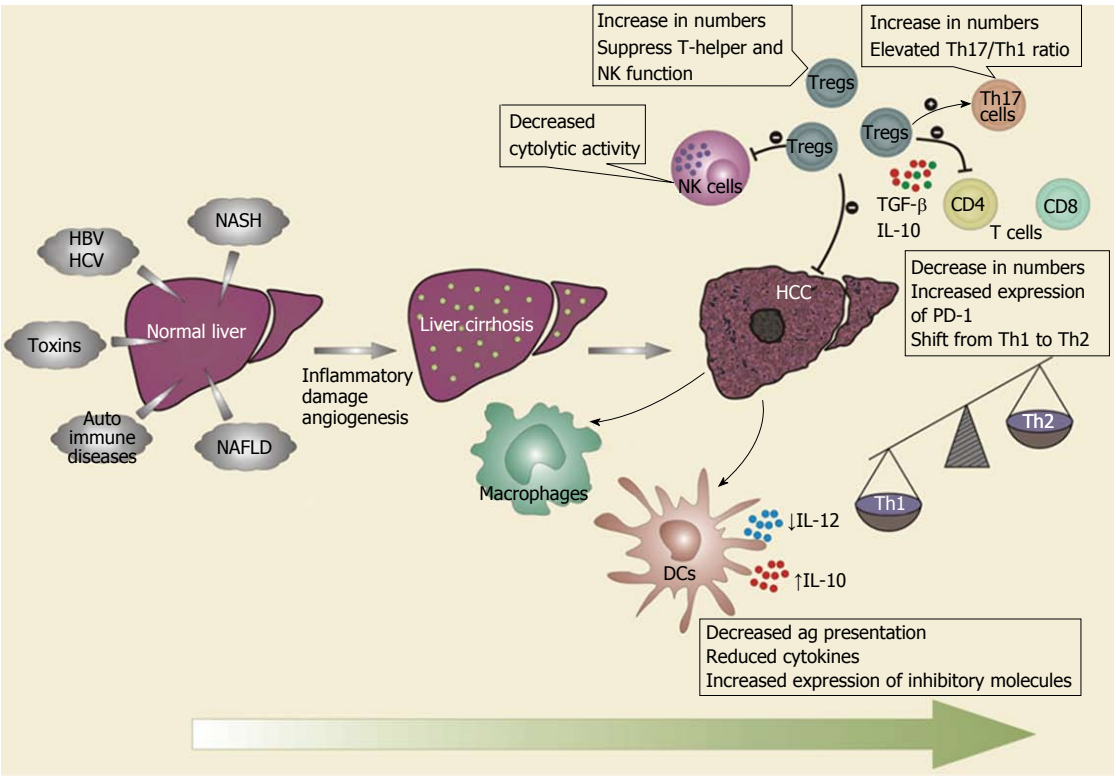

Figure 1 Role of immune cells in hepatocellular carcinoma. As the disease progresses from cirrhosis of the liver to hepatocellular carcinoma (HCC), the functions of various immune cells become dysregulated. Dendritic cells (DCs) lose their antigen presentation capabilities with the reduced secretion of Th1 cytokines. Macrophages differentiate into an "alternatively-activated phenotype" that generates a Th2-type immune response that promotes regulatory T cell (Tregs) recruitment and development. Natural killer (NK) cells have reduced cytolytic activities. T cells, both $CD4^+$ and $CD8^+$, decrease in numbers with attenuated function and increased expression of inhibitory receptors during HCC. Th17 cells increase in number and correlate with angiogenesis and poor-prognosis. Tregs exert negative effects on T cells, DCs, and NK cells, and may promote the differentiation of Th17 cells via immunosuppressive cytokines. There is shift in overall cytokine milieu from a Th1 to Th2 profile. HBV: Hepatitis B virus; HCV: Hepatitis C virus; IL-12: Interleukin 12; TGF: Transforming growth factor.

signifying the role of TAMs in the recurrence and metastasis of HCC[20,21].

Another heterogeneous population of cells called MDSCs, which are a subset of inflammatory monocytes, has been identified that comprises immature myeloid progenitors not already committed to any cell lineage[20]. They can exert inhibitory functions and regulate T cell responses through the up-regulated expression of several factors, such as free radicals, arginase activity, and production of TGF-β, thereby encouraging the induction of Treg cells[22]. Like typical monocytes, these cells express CD14 but have a lower or no expression of HLA-DR. An increased frequency of these cells has been reported in the peripheral circulation and tumor environment of HCC patients[23].

Similarly, neutrophils are a common inflammatory infiltrate in tumors that could also provide a prediction of poor survival in HCC patients, since their numbers correlated positively with the stage of cancer. Kuang *et al*[24] demonstrated that peritumoral stromal cells were fortified with neutrophil populations under the influence of Th17 cells through chemokines, like CXCL8, produced by epithelial cells. These neutrophils produce proteases like matrix metalloproteinase-9 in HCC tissues, promoting angiogenesis. Thus, neutrophils provide a connection between immune cells and angiogenesis, as well as promoting tumor growth.

NK cells

An exaggerated cytolytic population of NK cells serves as an immune invigilator in the liver microenvironment[25]. NK cells are cytotoxic and regulate the activity of other immune cells through the cytokines they release[26]. Under normal physiological conditions, NK cells mediate their functions in the liver *via* the production of "cytolytic granules" containing perforin, granzymes, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and interferon (IFN)-γ[27]. However, their functions are not completely imparted in the case of many cancers, including HCC. For instance, in HCC patients, a significant decrease in the CD56dim NK subsets in the peripheral blood has been reported as compared to healthy subjects[28]. A significantly lower proportion of these NK cell subsets exhibited reduced levels of IFN-γ, and cytotoxic potential also being reported in tumor regions compared with non-tumor regions in HCC patients[29]. Multiple mechanisms have been put forward to explicate the decreased functioning of NK cells and their association with cancer and cirrhosis of the liver, including fibrotic damage to NK cells[30], phagocytic uptake of NK

cells by activated hepatic stellate cells[31], and the up-regulation of inhibitory or down-regulation of activating receptors, respectively[32].

ADAPTIVE IMMUNE SYSTEM

T lymphocytes

T lymphocytes, both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells, are mostly considered to be significant players in inhibiting, impeding, and killing tumor cells. Their existence in cancer areas has been observed and correlated with a favorable prognosis in many cancers[33]. The IFN-γ produced during the Th1 immune response play a crucial role in the evasion and amelioration of HCC. In addition to helper T cells, the role of cytotoxic $CD8^+$ T-cells during HCC disease has been evaluated in many studies, where they were attributed a significant role in the killing of tumor cells.

In one study, it was reported that there was a significant decrease in $CD4^+$ T-cells in patients with liver cirrhosis and HCC, indicating their importance in facilitating malignancy among cirrhotic patients[34]. They likewise noticed a decreased ratio of helper T cells/suppressive T cells in the peripheral blood of patients with liver cirrhosis and HCC. Upon assessment of the genetic profile, a gene signature consisting of 17 immune related genes that changes the tumor microenvironment from a Th1 to a Th2 type milieu has been identified[20]. This foreshadows the development of venous metastasis in HCC, as well as impaired disease outcome, thereby indicating that progression of liver diseases is linked with a dysregulated cellular immune response.

Several mechanisms to deduce the immunosuppressive nature of T cells have been explained by many authors. Previous studies have shown increased levels of the soluble IL-2 receptor alpha chain, CD25 in the serum of cancer patients[35,36]. These studies have also shown a positive correlation between sCD25 and disease severity, serving as a surrogate indicator of survival and response to therapy[35]. The serum of HCC patients was evaluated, and revealed elevated levels of sCD25 as compared to normal, healthy individuals and patients with cirrhotic livers[36]. The authors observed an improvement in T cell responses after sCD25 depletion, suggesting that sCD25 is indeed involved in suppressing effector T cell functions.

Many human cancer cells express the ligand for inhibitory receptor PD-1. An up-regulated expression of its ligand, PD-L1, on intra-tumoral Kupffer cells and a concurrent increase in PD-1 expression on $CD8^+$ T-cells is detrimental in cancer[37]. Moreover, MDSCs were also found to have up-regulated expression of PD-L1, leading to functional exhaustion of effector cells through ligand-receptor interaction[37]. These data provide clues that strategies to block the PD-L1/PD-1 axis in HCC can increase tumor-specific immunity[38].

Another important effector subset of T helper cells are follicular T-helper cells (Tfh). These are important to B cells during germinal center reactions in secondary lymphoid tissues and function to support B-cell activation, affinity maturation, and isotype switching, leading to the generation of memory B cells and long-lived plasma cells[39]. Although only a few studies have focused on humoral immunity in HCC and its regulatory mechanisms, impairment of $CD4^+$ Tfh cells has been indicated to influence the development of HBV-associated HCC[40]. A decreased proportion of $CXCR5^+CD4^+$Tfh cells was found to be associated with HCC disease progression. Furthermore, these cell types were found to have an attenuated function with reduced secretion of IL-21, along with the inability to promote B cell maturation, and hence were suggested to be associated with low survival rates in HCC[41].

Regulatory T lymphocytes

Aside from anti-tumor cells that get functionally impaired during various cancers, there is another class of cells, termed Tregs, that express CD25 on their surface, along with the intracellular transcription factor forkhead box P3 (Foxp3), that have been reported to play a very important role in carcinogenesis[42,43]. Under normal physiological conditions, natural Tregs (nTregs) limit autoimmune reactions by suppressing self-reactive immune cells, and are also engaged in sustaining immunological self-tolerance and homeostasis.

It has been demonstrated in many studies that the number of a class of Tregs called induced Tregs (iTregs) increase in the peripheral blood and tumor infiltrating lymphocytes of patients with HCC[44]. Depletion of Tregs led to the manifestation of anti-tumor immune responses in this study in around 38% of HCC cases[45]. While the original investigations only demonstrated an increase in the frequencies of Tregs in patients with HCC[46], subsequent research was focused on the possible correlation of Tregs with disease progression and the clinical outcome of disease in patients[47]. It has been reported that the number of Tregs correlated with disease severity, as patients with advanced stages of HCC demonstrated a higher percentage of intrahepatic $CD8^+$Foxp3 regulatory T cells than patients in initial stages, suggesting that $CD8^+Foxp3^+$ regulatory T cells represent another immune-escape mechanism. Moreover, there was reduced infiltration of $CD8^+$ T-cells in tumors consequent to the abundant accumulation of Tregs in these areas as compared to non-tumor regions[48]. It has further been reported in another study that $FoxP3^+$ Tregs were highly amassed as activated cells expressing CD69 and HLA-DR (terminally differentiated subpopulation) in tumors where they could suppress T-cell proliferative capabilities and IFN-γ secretion by T cells[48]. Hence, it is suggested that the increased number of tumor-infiltrating Tregs fosters tumor progression and serves as a poor prognostic marker in HCC patients.

Furthermore, Tregs through their membrane-bound TGF-β, could also dampen NK cell responses by down-regulating NK group 2 member D expression and by participating in HCC progression[49]. Tumor-iTreg seem

to differentially regulate NK cell activity in the tumor microenvironment, as well as being endowed with abilities to modulate T-cell proliferative abilities and the functions of DCs *via* anti-inflammatory cytokines like IL-10 and TGF-β. In contrast with the nTregs, tumor iTreg cells interfere with NK cells activated with IL-2, while IL-2 independent activation of NK cells was augmented in the presence of iTregs[50].

Th17 cells

Ever since it became known that tumor cells of HCC, TAMs, and MDSCs are all capable of producing adequate quantities of IL-6 and TGF-β, it has been speculated that differentiation of Th17 cells in such an environment would be favored, especially in established tumor tissues. Coupled with extreme inflammatory conditions in growing tumors, an increased frequency of Th17 cells was more eminent in HCC tissues than non-tumor tissues, which positively correlated with microvessel density, a marker of tumor angiogenesis in tissues associated with poor endurance in patients with HCC[51]. Despite the positive correlation of Th17 cells with reduced survival in HCC cases, the role of these cells in HCC still remains incompletely defined. Some studies have recently suggested that IL-17 plays a dual role in tumor immunology; it can either promote anti-tumor cytotoxic T cell responses or foster angiogenesis of surrounding endothelial cells and fibroblasts facilitating tumor growth[52]. In HCC patients, increased levels of Th17 and Th1 cells were observed in tumor regions as compared to non-tumor regions, with the frequency of these cells being associated with overall disease-free survival[53]. Thus, an elevated Th17 to Th1 ratio may promote tumor progression and serve as a prognostic marker at the same time.

More recent studies have shown that an imbalanced proportion of Th17 cells and Tregs are also associated with cancer progression, but not much is known about the implication of this disproportion in cases of HCC[54]. The density of liver-infiltrated FoxP3$^+$ Tregs increased gradually from chronic hepatitis B infection to patients with atypical hyperplasia, then to HCC, while the density of Th17 cells and CD8$^+$ T cells in these cases trended towards a decrease as the disease progressed to HCC. In less differentiated HCC cases, the population of tumor-resident Tregs was lower, while the percentages of Th17 cells and CD8$^+$ T-cells were significantly greater. These findings indicate that Th17 cells and Tregs cooperate in the liver niche, thereby promoting cancer advancement.

NKT cells

NKT cells are a subset of T lymphocytes that have overlapping properties with both T cells and NK cells, expressing both the αβ T-cell receptor and many receptors of NK cells, and are a potent source of cytokines like IL-4, IFN-γ, and TNF-α. Depending on the diversity and extent of cytokines produced, their effects could be either beneficial or deleterious to the host. These cells recognize the non-polymorphic molecule CD1d, to which self and foreign lipid antigens are presented. These typical NKT cells, known as invariant NKT cells, act like a double-edged sword in cancer cases by promoting anti-tumor response *via* the activation of effector cells, while at the same time boosting the suppressor cell compartment and inducing tolerance[55].

Although NKT cells constitute a major population in the liver, their role in hepatocarcinogenesis remains incompletely understood. The frequency of NKT cells was increased in tumors, especially in HCC patients, with a gradual increment from blood to liver to tumor. A subset of these cells characterized by CD4 expression has been shown to accumulate in the tumor environment and is able to generate Th2 cytokines that inhibit the tumor-specific CD8$^+$ T-cell response[56], while the other subset, CD4-NKT cells, has anti-tumor effects and constitutes a key role in dampening the inflammatory response mediated by β catenin-driven hepatocarcinogenesis[57].

ROLE OF CYTOKINES AND CHEMOKINES

Dysregulated cytokine milieu

Hepatocytes express receptors for several cytokines, thus making them susceptible to their action. Consequently, cytokines are involved not only in the optimum functioning of the liver, development, and regeneration, but may also aid in the pathogenesis of liver cirrhosis, fibrosis, and HCC. The cytokine milieu in livers with metastatic HCC is skewed towards a Th2 profile, with an increase in levels of anti-inflammatory cytokines and a concomitant reduction in pro-inflammatory cytokines. This also highlights the importance of Th1-type immune response in inhibiting tumor relapse[58].

Th1 and Th2 cytokines

In Th1 cytokine levels, IL-2 is shown to have a direct correlation with prognosis in HCC patients, as the increased levels of IL-2 were associated with an increase in the number of CD8$^+$ T-cells[59]. Similarly, other Th1 cytokines like IFN-γ, IL-8, IL-15, and IL-18 have been indicated to correlate with invasiveness and metastasis during HCC[60]. Alterations in these cytokines may help to control or ameliorate carcinogenesis, as they are capable of changing the functional status of cells like NK cells and cytotoxic T lymphocytes[61].

The levels of Th2 cytokines, IL-4, and IL-5 were found to be high in the tumor microenvironment of metastatic HCC in patients with hepatitis B virus (HBV)-positive metastatic HCC, showing a shift from a Th1 to Th2 profile[21]. The causative factor associated with the switching of the cytokine balance is unknown, but factors produced by the tumor or microenvironment may play a role in tumorigenesis by polarizing cytokine production towards a Th2 phenotype.

Another cytokine released by Th22 cells[62] is IL-22, which has been found to be significantly elevated in HCC patients, suggesting an involvement in T-cell-mediated immunity in HCC. A direct relationship between the

levels of IL-22 and IL-17 in HCC patients indicates their interplay in the pathogenesis of HCC[63].

Pro-inflammatory and anti-inflammatory cytokines

TNF-α is an important mediator of inflammatory and autoimmune diseases and is strongly involved in the pathogenesis of HCC by promoting invasion, angiogenesis, and metastasis[64]. In many cancers, including HCC, the serum levels of TNF-α has been reported to be very high, which correlated with disease and nutrition status in these patients[65,66]. Although, in solid tumors, the levels of TNF-α were higher in normal tissues than in tumor cells, the serum levels were found to be lower in patients with HCC. Because of this discrepancy, the precise impact of cytokines associated with liver cancer development remains unclear[67]. TNF-α is also known to stimulate the expression of the negative co-stimulatory molecule B7 homolog 1 or PD-L1 on macrophage surfaces, thus suppressing the $CD8^+$ T-cell anti-tumor immune response[38]. The principal downstream mediator of pro-tumoral TNF-α activity is nuclear factor κB (NF-κB), whose target genes are involved in cell proliferation and survival[68]. TNF-α is also notably induced by NF-κB in a positive feedback loop.

Higher production of IL-1β may help increase the production of other cytokines, such as IL-2, IL-6, and TNF-α, and trigger the complex immunological processes to eliminate the virus in cases of hepatitis-induced HCC. Interestingly, besides its major role as a pro-inflammatory cytokine, IL-1β has been implicated as an important factor for tumor growth. Several independent lines of evidence have also suggested that genetic polymorphisms within the *IL-1β* gene are associated with gastric cancer and HCC induced by HCV infection[69,70]. Moreover, supplementing cytokines like TNF-α, IL-1β, or IL-18 has been shown to induce growth of $CD8^+$ T-cells and induce TRAIL in many HCC cell lines, thereby contributing to tumor evasion[71].

The most studied anti-inflammatory cytokine in HCC is IL-10, which has been shown to be increased in HCC tumors *vs* non-tumorous tissue adjacent to the tumor and tissues of healthy cohorts, respectively[72]. These studies suggest that an increase in IL-10 in conjunction with other Th2 cytokines correlates with progression. Another multifunctional inflammatory cytokine, IL-6, which is produced mostly by resident macrophages, was found to be linked with poor prognosis in HCC patients[73]. IL-6 exerts its oncogenic activity by triggering downstream signal transducer and activator of transcription 3 and extracellular-signal-regulated kinase pathways, which in turn control target genes involved in both cell proliferation and survival. It has been found that IL-6 levels and receptor expression were raised in a number of cancers, including HCC, where it may contribute to tumor progression[74]. Recently, in a study carried out to investigate the use of novel serum biomarkers for predicting the recurrence and survival of patients with HBV-related HCC, low serum IL-6 level, low platelet count, and low serum albumin level were found to be independent prognostic factors for disease-free survival in these patients[75]. IL-37, a recently recognized anti-inflammatory cytokine has been shown to suppress cells of the innate immune system[76]. The study indicates that in HCC specimens, the expression of IL-37 was found to be decreased in tumor tissues and its expression level was negatively related to tumor burden and survival improvement.

Hence, it could be concluded that cytokines regulate the microenvironment of immune cells with allied and opposing roles, involving different signaling pathways to affect the course of HCC disease.

Chemokine ligand-chemokine receptor axis

Chemokines are known to direct lymphocyte recruitment into liver tumors expressing the corresponding chemokine receptors[77]. The CXCL12-CXCR4 axis is regarded to be critical as a factor regulating tumor growth and progression during HCC. Previous studies have depicted higher expression of CXCL12 and CXCR4 in HCC specimens than the surrounding tissues[78]. It has been demonstrated in different studies that CXCR4 and CXCL12 may play a significant part in HCC metastasis and invasiveness of the tumor[79,80]. A significant correlation was observed between CXCR4 expression, tumor progression, metastasis, and a decreased survival rate[80]. However, the lack of a loss of function mutation of the tumor suppressor gene p53 gene on CXCR4 expression in HCC indicated yet another unidentified mechanism[81].

However, an ambiguity as to whether CXCR4-CXCL12 actually promotes tumor growth as a down-modulation of CXCR4-CXCL12 expression in HCC, both *in vitro* and *in vivo* has been reported, where CXCL12/CXCR4 also lacked an association with death and HCC recurrence[82]. Therefore, although it appears that the CXCL12-CXCR4 axis is indispensable in HCC, its precise role still remains paradoxical in this disease. The possible involvement of the CCL20-CCR6 axis in HCC has been suggested because of the significantly up-regulated expression of both CCL20 and its chemokine receptor CCR6 that has been observed in HCC tissues with different rates of tumor progression[83]. Although the role of fractalkine (CX3CL1) and its receptor CX3CR1 in HCC indicated a role in the regulation of immune response, the relationship of between the fractalkine-CX3CR1 axis and HCC is as yet unclear. According to recent studies, the fractalkine-CX3CR1 axis is critical in the diagnosis of HCC, as it can regulate both the immune response and the cell cycle of HCC[84].

Furthermore, the expression levels of some chemokine receptors like CCR5, CCR6, and CXCR3 on the surface of peripheral lymphocytes of HCC patients was reduced, while the expression of these receptors on tumor-infiltrating cells was higher, suggesting a role of these chemokine receptors in controlling the trafficking of effector T cells to the tumor regions in response to the corresponding chemokines[85]. In addition to this, the expression levels of CXCR3 have been reported to be particularly high in tumor infiltrating cells, as compared

Table 1 Summary of the status of various immune components in hepatocellular carcinoma

Immune component	Status in HCC	Ref.
Dendritic cells	Decreased antigen presentation, decreased numbers, impaired functions	[12,13]
Macrophages	Poor antigen presentation, activated Th2 immune responses, promoted Tregs	[17,18,20]
Myeloid-derived suppressor cells	Exerted suppressive functions through free radicals, arginase activity, and TGF-β	[21,22]
Neutrophils	Promoted angiogenesis through metalloproteinase-9	[24]
NK cells	Decreased numbers, low cytolytic activity	[26,28]
T lymphocytes	Decreased frequency, fewer Th1 cytokines, increased expression of inhibitory receptors	[36,37]
Tregs	Increased frequency, suppressed T-cell proliferation and IFN-γ secretion, inhibited NK cell responses	[42,48,86]
Th17 cells	Increased numbers, incompletely defined role, correlated with disease progression	[51,52]
NKT cells	Dual roles, increased frequency, promoted Th2 cytokines	[55,56]
Th1 cytokines	Decreased in tumor microenvironment, induced $CD8^+$ T-cells	[59,61,87]
Th2 cytokines	Increased levels, correlation with tumor progression	[21]
Proinflammatory cytokines	Involved in pathogenesis of HCC	[65,69]
Anti-inflammatory cytokines	Increased in HCC, correlated with progression	[72,73,76]
Chemokine-receptor axis	Tumor progression and metastasis	[78,83,84]

HCC: Hepatocellular carcinoma; NK: Natural killer cells; Tregs: Regulatory T cells; Th17: T helper type 17; NKT: Natural killer T; TGF: Transforming growth factor; IFN: Interferon.

to non-tumor infiltrating cells, implying that lymphocytes preferentially migrate to the tumor tissue rather than the surrounding non-tumor regions. This increased expression was negatively correlated with tumor burden and the stage of cancer. The literature citing the role of various immune components in HCC is summarized in Table 1.

GAPS IN EXISTING KNOWLEDGE

Insights into the immune signaling pathways are being provided by recent studies analyzing the role of immune effector cells. However, a complete understanding of many immune components, such as NKT cells, gamma delta T cells, and the role of many cytokines and chemokines, has not yet been achieved. It is generally believed that T lymphocytes play a protective role in inhibiting tumor growth and development, while TAMs, MDSC, Tregs, Th17 cells, and their associated cytokines (IL-6, TNF-α, IL-1β, IL-23, and TGF-β) may play important roles in promoting the growth and survival of cancer. However, defining their roles as pro-tumor or anti-tumor still requires caution. It is also unclear as to how TAMs and TGF-β regulate the generation and function of Tregs in the development and establishment of the solid tumor microenvironment. Of further importance is understanding whether TGF-β production preferentially induces Tregs or promotes the development of Th17 cells within the tumor microenvironment. Further research into better understanding the balance between all immune components at all stages of carcinogenesis is essential for the development of effective cancer therapies that target or utilize immunological mechanisms.

Recent observation of many solid tumors suggests the use of checkpoint inhibitors that decide a balance between co-stimulatory and inhibitory signals in inducing a strong anti-tumor response that needs to be evaluated in HCC. Tumor vaccines and therapeutic agents for targeting various checkpoints represent some novel strategies for inducing immune resistance. These combinatorial approaches induce tumor regression in patients that would not have responded to either treatment alone. Strategies to deliver genetically modified T cells into the tumor microenvironment, such as *via* a hepatic artery, are underway and being evaluated in clinical trials that have already proven successful in the treatment of other cancers[88]. Novel epitopes specific for tumor-associated antigens should be designed using high throughput "omics" technology with the aim to induce anti-tumor $CD4^+$ and $CD8^+$ T-cell responses. In this context, high resolution mass spectrometry has been used for directly sequencing peptides presented by HLA molecules from tumor cells so as to identify naturally processed class Ⅰ and Ⅱ tumor-associated peptides[89]. Combining key components of the tumor microenvironment, as compared to chemotherapy alone, would improve the clinical outcome. Finally, therapeutic agents capable of reversing the immunosuppressive nature of HCC tumors *via* administration alone or in combination with other modalities will be critical in optimizing clinical outcomes for HCC patients.

CONCLUSION

Since HCC accounts for 90% of all liver cancers and is usually multifocal at the time of diagnosis, treatment is difficult and affronted with a higher recurrence rate in these patients. The incidence of the disease is accelerating at a regular rate and will likely increase further over the coming years. Hence, in this context, there is an imperative demand for newer and better therapeutic strategies to combat this predicament. This requires a fuller discernment of the function of various components of our immune system and how they interplay in creating immune responses against tumors. Immune suppression is predominantly mediated by cytokine secreted in the local milieu by Tregs that down-regulate the effector and cytotoxic activities of $CD8^+$ T-cells and NK cells. The antigen presenting functions of DCs are also affected due to the expression of several

inhibitory receptors that further suppress the functions of helper T cell. TAMs and MDSCs contribute to the ongoing inflammation and participate in the activation of a Th2 immune response that favors Treg recruitment and development, thus promoting angiogenesis. These cell types can help in the differentiation of Th17 cells that also infiltrate the tumor microenvironment, and correlate with poor survival in HCC patients; however, their roles still remain incompletely defined. Similarly, despite being the predominant population in the liver, the role of NKT cells in hepatocarcinogenesis remains to be completely elucidated. Soluble factors, including cytokines and chemokines, play a crucial role in immunosurveillance and immunoregulation. The cytokine milieu in livers with metastatic HCC is skewed towards a Th2 profile, with a concomitant decrease in pro-inflammatory cytokines. The roles of many cytokines like IL-22 have recently been deciphered in HCC, which adds to the current knowledge about the milieu of liver tumors. The chemokine ligand-chemokine receptor axis plays a role in regulating the differential recruitment of effector T cells to the tumor and the interconnections between different axes, as not just a single axis should be surmised. Future studies are warranted to understand the complexity of interactions between these immune cells to potentiate the immune system and for the designing of newer immune-therapeutics against HCC.

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P- Reviewer: Calvisi DF, Chua MS, Geller DA, Simonovic SZ
S- Editor: Ma YJ **L- Editor**: Rutherford A **E- Editor**: Liu SQ

World Journal of Hepatology

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Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4254/wjh.v7.i17.2091

World J Hepatol 2015 August 18; 7(17): 2091-2099
ISSN 1948-5182 (online)

MINIREVIEWS

Addiction specialist's role in liver transplantation procedures for alcoholic liver disease

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Author contributions: Dom G and Peuskens H both contributed to this paper.

Conflict-of-interest statement: None.

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Received: April 26, 2015
Peer-review started: April 28, 2015
First decision: June 25, 2015
Revised: July 24, 2015
Accepted: August 10, 2015
Article in press: August 11, 2015
Published online: August 18, 2015

Abstract

Although liver transplantation (LT) is performed increasingly for patients with end-stage alcoholic liver disease (ALD), the topic remains controversial. Traditionally, the role of an addiction specialist focused on the screening and identification of patients with a high risk on relapse in heavy alcohol use. These patients were in many cases subsequently excluded from a further LT procedure. Recently, awareness is growing that not only screening of patients but also offering treatment, helping patients regain and maintain abstinence is essential, opening up a broader role for the addiction specialist (team) within the whole of the transplant procedure. Within this context, high-risk assessment is proposed to be an indication of increasing addiction treatment intensity, instead of being an exclusion criterion. In this review we present an overview regarding the state of the art on alcohol relapse assessment and treatment in patients with alcohol use disorders, both with and without ALD. Screening, treatment and monitoring is suggested as central roles for the addiction specialist (team) integrated within transplant centers.

Key words: Liver transplantation; Alcohol use disorder; Alcoholic liver disease; Relapse; Addiction specialist

Core tip: Liver transplantation is performed increasingly for patients with end-stage alcoholic liver disease. Assessment of a patients risk on relapse in alcohol use after transplantation and helping patients to achieve and maintain abstinence are crucial within this process. The addiction specialist's input is essential and needs to be integrated within the transplantation team. Ideally a multidisciplinary approach is offered to the patients, including addiction psychiatrist, behavioral therapist and social worker following up the patient before and after transplantation.

Dom G, Peuskens H. Addiction specialist's role in liver transplantation procedures for alcoholic liver disease. *World J Hepatol* 2015; 7(17): 2091-2099 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2091.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2091

INTRODUCTION

Alcohol use disorders (AUDs) are highly prevalent and devastating disorders. Within the general population about one in five people meet the criteria for an AUD in their lifetime[1]. The net effect of alcohol consumption on health is detrimental, with an estimated 3.8% of all global deaths and 4.6% of global disability-adjusted life-years attributable to alcohol[2]. A large portion of these effects is due to negative consequences of (excessive) alcohol use on the liver. Indeed, there exist a direct, exponential, relationship between the amount of alcohol consumed on a population level and the prevalence of chronic, end-stage liver disease (*e.g.*, cirrhosis)[3,4].

Liver transplantation (LT) is increasingly used as a life-saving intervention for patients with end stage alcoholic liver disease (ALD). Currently between 30% to 50% of all LTs in Europe and about 17% in the United States, are performed in the context of ALD[5-7]. Importantly, post-operatively between 30% and 50% of the patients relapse in any alcohol use and 20% to 25% of them relapse in heavy alcohol use[8]. Relapse in long during and excessive alcohol use after LT increases the risk on allograft damage and mortality[9,10]. Thus both from the point of view of patient safety and within the context of chronic low allograft availability, efforts are justified towards using valid screening procedures to identify the most suitable candidates and offering treatment to help patients to (re) gain and maintain sobriety[7]. Ideally, both therapeutic aspects, *i.e.*, screening for relapse risk and offering personalized addiction treatment, should be integrated in a patient's treatment plan. However, within many current (pre) transplantation procedures, the focus of attention remains on screening procedures, while much less effort is invested to engage patients in continuous addiction treatment[11].

In this review we provide first an overview of the current state of the art on AUDs as a whole and compare it to the specific situation of patients with ALD as candidates for LT. Pertinent questions are whether ALD-LT patients are different from general AUD patients and whether there are differences in alcohol use outcome and treatment modalities between both groups? Finally, we focus on the role of addiction specialist in screening and treatment of patients with ALD within the context of LT procedures.

TWO TYPES OF AUD PATIENTS

The natural course of AUD varies vastly; from very positive outcomes reported in general population samples meeting alcohol abuse or dependence criteria to very negative outcomes in treatment seeking patients in criminal justice settings[12]. Overall, most affected individuals recover naturally without any formal type of treatment, and approximately 70% of individuals go into remission within three years[13,14]. In accordance, longitudinal studies in general population samples show low AUD relapse rates, *i.e.*, 5.6%, 9.1% and 12.0% at respectively five, ten and twenty years of follow-up[15]. In contrast, people seeking treatment for AUD' s represent a much smaller, but more vulnerable group characterized by a lower resilience, higher risk for relapse, more problems in different life domains, and overall a more negative course of the disorder. In this group AUDs develop into a chronic relapsing disorder and relapse in heavy or dependent drinking occurs in over 50% of patients. This latter group of patients, *i.e.*, high psychosocial co-morbidity, typically presents in addiction treatment programs, while the former group, *i.e.*, low psychosocial co-morbidity, is more prevalent in gastroenterology departments due to the somatic alcohol related consequences[16,17].

Regarding LT, current enrollment procedures appear to make for a consistently more favorable outcome in LT patients in comparison to alcohol treatment seeking populations, with five and ten year follow up show relapse rates (any alcohol use) of respectively 20% and 30% in LT patients, and more recent studies even lower[18]. In addition, Weinrieb *et al*[19] suggest that ALD-LT candidates differ substantially from AUD patients within standard addiction programs. ALD-LT patients hold their medical health and transplant management to be a priority over addiction treatment, perceiving less of a need for addiction counseling. Indeed, many do not look upon themselves as being addicted and do not identify with the prototypical "chronic relapsing alcoholic" image. As a consequence, demands for addiction counseling and regular alcohol monitoring are frequently experienced as offensive by these patients, often leading to defensive reactions. However, differences between these two types of patients may be the result of a selection bias, as patients with more complicated, behavioral and psychiatric symptom cluster are often screened as "high at risk" for relapse and in current procedures not included in a subsequent LT trajectory.

PREDICTING RELAPSE IN AUDS

Relapse prediction provides the opportunity not only to identify specific patients groups with poorer outcomes, but also - and relevant to any clinician, helps identify areas to target in treatment[20]. Research on this topic is currently highly topical within the whole of the addiction field.

Predictors of relapse alcohol in non-ALD alcohol patients

Most studies looked into demographic and clinical variables. Overall, dependence severity, psychopathology ratings, alcohol-related self-efficacy, motivation, cognitive impairments, and treatment goal, are all associated with relapse risk[15,20-22]. In addition, the duration of abstinence in itself is a predictor of future relapse. Indeed, for many afflicted individuals stable

Table 1 Assessment alcoholic liver disease patients for liver transplantation

Dimension	Variable
Severity alcohol use (disorder)	Amount of alcohol use and baseline alcohol use (TLFB)
	AUD diagnosis severe (DSM5)
	Family-history AUD
	Age-at-onset AUD
	Duration
Abstinence	Duration pre-LT abstinence
Treatment indicators	Earlier treatments for AUD
	(Longstanding) periods of abstinence
	Compliance medical treatment
Co-morbidity	Psychiatric
	Other substance (mis) use (Illicit drugs, tobacco)
Cognitive	Memory
	Executive
Social	Partner and family
	Living in supportive, clean, circumstances
	Employment
Personal	Motivation
	Self-efficacy

TLFB: Timeline follow-back; DSM5: Diagnostic and Statistical Manual, 5th ed (APA, 2013); AUD: Alcohol use disorders.

remission of AUD is to be expected only after about five years of abstinence[23]. Taken together, although many clinical variables have been identified, not one single one stands out as decisive. Results show for each of them a low to moderate predictive power and are not always consistent. This might reflect the fact that almost none of these studies take the heterogeneity of AUD patients into consideration. Furthermore, clinical variables may not be specific enough and might not relate directly with the underlying pathogenic processes. Recently, focus of research is shifting and consensus is growing that neurocognitive measures might help identifying patients with a high risk for relapse[24,25]. In addition, functional brain imaging markers, cue or stress-reactivity paradigms, are starting to reveal not only the underlying vulnerability mechanisms, but allow predicting relapse in alcohol addiction[26-28]. It can be argued that imaging biomarkers, for practical and financial reasons, are not of use in a standard treatment program. In contrast, a small but increasing number of treatment centers start to adopt the use of neurocognitive measures for better profiling their patients with respect to outcome prediction[29,30].

Taken together, research into relapse prediction is still ongoing, and up to now has not delivered a set of easily measured variables that can, reliably, predict relapse on an individual's basis. Overall, clinician's judgment, helped by some clinical and neurocognitive measures, remains the core of the assessment process.

Predictors of relapse after LT

Although both the number of studies and the sample sizes are (much) smaller than in AUD studies, research into relapse prediction in LT patients identified the same set of clinical variables that are associated with relapse in AUD patients[31] (Table 1). Typical for the LT context is the importance that has been given in most screening-procedures to pre LT abstinence period as a predictor for relapse. This, so called 6-mo abstinence rule has recently come under discussion. Indeed, although abstinence duration is one of the clinical predicting, albeit moderately powerful, relapse-predicting factors, the specific six-month minimum period is not supported by the data. In addition, many patients with end stage ALD simply do not have that time and many will die in the process of bridging these 6-mo. Unquestionably, a substantial period of abstinence is warranted allowing for a stable abstinence and recuperation of the liver functionality (EASL Guideline, 2012). Given that recuperation of liver functioning is not expected after more than 3 mo of abstinence, prolonging this period likely results in a higher patient mortality risk, which is not compensated by a gain in power when assessing relapse risk after ALD. Taken together, accepting abstinence periods of less then 6 mo within LT screening procedures may include a small increase of risk on post-LT relapse in alcohol use. However, this must be balanced with the other clinical risk factors. Shorter abstinence periods cannot be used as a single criterion for non-inclusion. Instead this should be considered an indication to intensify addiction treatment, in order to reduce relapse risk.

Concluding, when evaluating an ALD patient in view of LT, it is important to acknowledge that currently no single clinical variable can be used when assessing the relapse risk. This implies that within the context of the LT screening procedure the addiction specialists (or team), needs to rely on a comprehensive assessment, evaluating a set of different, *i.e.*, clinical, demographic, and social, variables. Recently, some groups have suggested scoring systems[31-33], incorporating a fixed set of variables and related scoring. Although of interest, a systematic, multi center evaluation into the validity with respect to relapse prediction of these scoring systems is currently missing.

TREATMENT OPTIONS

AUDs

One of the most important problems regarding AUDs is the extreme treatment gap. Indeed, within Western countries only about 10% of the potential patients receive any form of alcohol treatment[34]. Decreasing this gap would be the sole most important intervention from the point of view of population health. Indeed, when a patient can be reached, a variety of (moderate) effective treatments can be offered.

Psychosocial interventions: Many types of, mostly behavioral, therapies have been developed and tested with well-performed studies[35]. Overall these studies show that these treatments are effective in reducing alcohol use, although, comparable with pharmacological treatments the effect sizes are moderate. Of importance, short interventions are very effective for the

large majority of individuals with a heavy or hazardous, but not dependent drinking-pattern. Next, in addition to (cognitive) behavioral therapies, more complex, multi-target interventions (*e.g.*, Community Reinforcement Approach) have been developed for patients with a high problem severity and earlier treatment failure.

Of interest, treatments for AUD are increasingly offered in online formats[36]. Specifically, complex attentional- and approach bias modification strategies have proven significant results both as stand alone and on top of treatment as usual procedures. Remarkable, these interventions not only improve alcohol outcome, but have recently also shown to change underlying neurobiological cue-reactivity pathways[37,38]. The advantages of online treatment modalities are multiple; low barrier for patients with limited mobility or time availability, anonymity, and lower cost especially when (quasi) fully automated.

Pharmacological interventions: A small number of medications are registered for the treatment of AUDs (disulfiram, naltrexone, acamprosate, nalmefene) while some other medications have shown promise mainly in short-term studies (*e.g.*, topiramate, GHB, baclofen, and gabapentin)[12]. While most of these treatments take abstinence as main treatment goal, recently interest is growing for a reduction of alcohol use as a valid treatment goal[39,40]. Overall, effect sizes of pharmacological treatments are moderate, *i.e.*, on the same level as antidepressants for depression, and there is no treatment that seems to fit all patients. In search for a more personalized approach in patient-treatment matching, pharmacogenetics seems promising[12,41].

Liver transplant patients

Compared to the number of studies on screening and relapse prediction, a remarkable limited number explore the effect of addiction treatment interventions in this population.

Psychosocial interventions: Some studies showed that offering treatment in the pre-LT (waiting list) period was associated with reduce the number of patients relapsing in (any) alcohol use during the waiting period and after LT[7,19,42,43]. Of importance, successful treatment effect was reported in an other study, only in the subsample that engaged in treatment both before and after LT, underscoring the importance of post-LT treatment[44].

Finally, Addolorato *et al*[11] found that AUD treatment offered by addiction specialists integrated within the transplant team had superior results, *i.e.*, less alcohol recidivism and lower mortality rates, than treatment offered by an addiction specialist outside the transplant team.

Pharmacological interventions: When considering pharmacological interventions for AUD treatment in ALD patients, one needs to take the severe liver dysfunction into consideration. Currently, only a very limited number of studies explored the feasibility, safety and effectiveness relapse-prevention medication in these patients[7]. As of consequence, the use of alcohol medication pre LT or in patients with liver cirrhosis is extremely limited. Recently baclofen, which is not metabolized in the liver, showed both safety in use and positive effect (continuous days abstinence, craving) in patients with end-stage ALD[45]. Currently, no studies have been done using pharmacotherapy for alcohol relapse in post-LT patients. Based upon their pharmacological profile, specifically those medications that are not metabolized in the liver can be considered as potential candidates (*e.g.*, acamprosate, baclofen, topiramate).

MONITORING

During both the pre- and the post LT period, a close monitoring of alcohol use is needed, as an integrated part of the psychosocial follow-up. In addition to self-report and collateral information, the importance of biomarkers is increasingly recognized (see for review Vonghia *et al*[46]). Traditional alcohol biomarkers such as gamma-glutamyltransferase are not recommended in ALD patients because they will be elevated as a result of the liver damage itself. They could provide some information in post-transplant patients, however, as also within non-ALD alcohol patients, they have low sensitivity (30%-60%) and specificity (60%-95%). Other often-used biomarkers in blood (MCV, ALT, and AST) also have low sensitivity (< 50%) and specificity (60% to 95%), and are also confounded by liver damage itself[47,48].

Carbohydrate-deficient transferrin (CDT) is more specific for heavy (from 5 to 6 standard drinks per day for several days) alcohol use and will be elevated for about two weeks after the drinking bout. However, in pre-transplant ALD patients, CDT lacks specificity. However, as a post-transplant measure it has value as an indicator of heavy alcohol use. In comparison with other biomarkers, CDT would be a biomarker that is less affected by false positive results due to liver disease[49,50].

Recently, several recent studies suggest a promising role for using ethylglucuronide in hair samples (hEtG) as a biomarker for alcohol use detection. Indeed, traditional biomarkers for alcohol use in blood and urine allow only limited detection windows (hours to days). In contrast, hair serves as a long-term storage of EtG, covering much larger time periods (months). In addition, collecting hair samples is non-invasive and samples can be saved easily and for longer periods. Increasingly, validated cut-off scores are available, that allow distinguishing between chronic, excessive, moderate alcohol use and abstinence (Society of Hair Testing; www.soht.org)[51-53]. Specifically within the context of monitoring LT patients, the use of hEtG has proved to be a highly specific and practically implementable biomarker that is superior to traditional

markers[50,54-56].

ETHICAL CONCERNS

End stage liver disease (ESLD) has a high mortality ratio and often, liver transplantation is the only, life-saving, therapy. For many years, given on the one hand the imbalance between organ availability and demand, and on the other hand the continuum "moral" attitude (*i.e.*, "not a disease but a weakness of will") towards individuals with alcohol problems, controversy existed and sometimes remains, whether ALD was an indication for LT. This controversy contrasts with the accumulating data showing that: (1) similar and even better survival rates than LT for ESLD of other etiology (*e.g.*, hepatitis C); (2) low alcohol relapse rates compared with non-ALD alcohol dependent patients; and (3) limited alcohol use after LT is not associated with severe negative consequences. One of the consequences of this controversy is that when evaluating a patient, procedures and protocols are mainly focused on "screening out" those at risk for relapse in alcohol use, instead of focusing on developing tools and methods to help patients gain and maintain sobriety[7]. In addition, alcohol outcome goals are used at their most severe, *i.e.*, complete abstinence and the 6-mo rule. These goals are more severe than in standard addiction treatment programs where the focus is increasingly put on shared decision-making concerning treatment goals, reduction of use to safe levels, and enhancing continuous motivation. However, the research data do not support the need for this degree of severity in treatment goals in LT patients. Of interest, this "selective" focus on alcohol-abstinence is all the more remarkable when one notices the much lesser attention on, potential also harmful, health-behaviors, *e.g.*, abuse or intoxication acetaminophen, intravenous drug use with hepatitis and continuing cigarette smoking. Several reasons may be at play maintaining this alcohol-controversy. First, financing bodies might keep up with these high barriers, in the hope of containing the number of these, indeed, expensive treatments. Second, concerns might rise that lowering the threshold would be poorly perceived by the general public, hence risking decrease in willingness to donate organs[57,58]. Finally, continuing moral and stigmatizing thinking about addictive behaviors might still play an important role both within the general public as within the medical profession, resulting in poor professional and patient-lobbying towards changing the procedures and financing contingencies.

Taken together, individuals with alcohol remain frequently negatively regarded upon. Even in highly specialized medical settings such as hepatology and transplant centers, the risk on stigmatizing attitudes and consequent actions is not illusionary. An important advocacy-role for the addiction specialists (team) is to constantly be alert for and act on signs of possible discriminatory behaviors and procedures, so that ALD patients receive the same quality of care and respectful context that every patient is entitled to.

ROLE OF THE ADDICTION SPECIALIST (TEAM)

Addictions specialists (team) have important roles during the whole process of the ALD-LT procedure (Table 2). Given the complexity and diversity of the core services to be provided, as described infra, ideally this work is taken on by a multidisciplinary addiction specialist team. Although economic barriers and possibilities may differ widely between countries, team composition should at least contain a psychiatrist, psychologist-psychotherapist, and social worker, all trained in addiction work. Their services need to be offered as an integrated part of the transplant program[11].

Screening

The addiction specialist role is a thorough screening, leading to an assessment and assignment to risk categories. This is a comprehensive assessment including interviews with patients, family and relevant others. Given that none of the known risk factors is conclusive, the final decision is by definition based upon a careful balancing of all elements. Different dimensions need to be assessed (Table 1), *i.e.*, individual (motivation, treatment compliance), co-morbidities (psychiatric and other substances), cognitive, AUD severity and treatment history, and social support systems. Categorizing patients will help to allocate them to better matched treatment and follow-up procedures. Broadly two groups can be identified. First, patients with low psychosocial co-morbidity. Individuals with this profile, tend to have a positive course of their AUD, low-relapse risks, and a good change on stable abstinence and/or reduction of drinking to save levels. They can be allocated to less intensive addiction treatment, *e.g.*, short interventions, aimed at enhancing motivation, counseling, self-help and monitoring. A second category, *i.e.*, "high risk", is those people that accumulate risk factors for a negative, chronic relapsing nature of their AUD. This group is within the current LT procedures often excluded. A much more intensive addiction treatment is needed. Whether this should be mandatory for all patients is a matter for discussion, but mandatory treatment needs to be considered with poorly compliant patients.

Both within the screening-assessment procedures as to the monitoring during waiting list periods, one of the most challenging questions remains how much information is shared between the addiction team and the transplant team. Indeed, a high level of confidentiality is needed in the relation between the addiction specialist and the patients, facilitating an open sharing, necessary for treatment and growth of motivation. On the other hand, when relapse risks are high some information needs to be communicated allowing a balanced discussion between transplant and addiction team on very difficult questions of candidacy

Table 2 Role of the addiction specialist (team) in the screening, treatment and monitoring liver transplantation candidates

Screening	Category	Waiting list period	LT	Post-LT physical rehabilitation	Long term follow-up (> yr)
Following items need to be surveyed to decide upon which category patient will enter treatment traject:	"Low risk"	Who: Addiction treatment team integrated within transplant/ hepatology department What: Motivation enhancement and relapse prevention strategies		Psychosocial support patient and family	"Low intensity follow-up" (1) Who: Addiction treatment team integrated within transplant/hepatology department; or, addiction counselor in the living area of patient (2) What: Motivation enhancement Counseling Relapse prevention Anticraving medication: baclofen/acamprosate
	Monitoring alcohol, drug and use tobacco use				
	"High risk"			Psychosocial support patient and family	"High intensity follow-up"
		Who: Addiction treatment team integrated within transplant/ hepatology department What: Motivation enhancement and relapse prevention strategies			(1) Who: Comprehensive addiction treatment program/ care provide/living area patient (2) What: Comprehensive integrated treatment including different treatment options that can be put in function of specific patient needs: Complex behavioral interventions helping patients to control alcohol and comorbid substance (drug, nicotine) use and prevent relapse: CBT, CRA Diagnosis and psychosocial treatment interventions psychiatric co-morbidities Pharmacotherapy directed at craving control (baclofen, acamprosate, nalmefene) Availability of settings: assertive outreach, (semi) residential programs

CBT: Cognitive behavioral therapy; CRA: Community reinforcement approach.

for transplantation. As yet no clear-cut solution for this dilemma is at hand. However, it is of utmost importance that it is very transparent for the patient and family what is communicated and what the consequences can be.

Treatment (coordination)

Pre-LT: Most patients during the waiting-list phase are physically very ill and often have cognitive impairments. Treatment at this phase should focus on the one hand on psychological support for patient and family, enhancing motivation for abstinence, and on the other hand permanent monitoring of alcohol use. In most settings, patients will have frequent contacts with the hepatology/transplant center, so the addiction specialists (team) of the center are best placed to engage in this follow-up.

Post-LT: The first phase after LT is usually a period of medical-somatic revalidation, in which for most patients alcohol use is no issue. Thus, a low-intensity addiction follow-up, with monitoring of alcohol use will be enough. Risks on relapse (and associated treatment non-compliance) will increase when physical recuperation allows the patient taking up a more active life style. At this point addiction treatment interventions need to intensify. Depending whether a patient lives close by or farther away from the transplant center, addiction specialists (team) can or deliver treatment themselves, or function as coordinators, organizing a treatment program within the patient's region.

For patients classified as low-risk, usually a none-intensive standard alcohol treatment can be put in place; counseling aimed at motivation enhancement and coping skills relapse prevention and continued monitoring alcohol use. Self-help groups like AA can be helpful, though not many of these LT patients identify to this degree with the label "alcoholic", putting a barrier for engaging in self-help.

Patients classified as high risk will need a comprehensive and integrated treatment program allowing the (simultaneous) use of different treatment interventions, targeting the often multiple problem domains, *i.e.*, psychiatric, (other) substance use, cognitive, and social. If needed, assertive outreach and (semi) residential slots should be available. This type of comprehensive, specialized addiction treatment is often beyond the possibilities of the addiction specialists (team) within a transplant center. Thus, its role in this context is helping to organize and coordinate this program in close collaboration with an addiction-center and to ensure liaison with the transplant center.

Monitoring

Throughout all the treatment process continued moni-

toring of alcohol use is warranted. Addiction specialists should carefully interpret data from self-report, collateral information biomarkers. Findings can be used as feedback for patients helping them to improve compliance and abstinence. It is still an open debate whether data from monitoring should be shared with members of the transplant team. Specifically during the waiting-list period, patients will be afraid that these will be used against them, so openness might be jeopardized. It might be wise to agree in the treatment plan that only addiction specialist are allowed to follow-up monitoring.

CONCLUSION

From a broader addiction specialist point of view, ALD patients that are LT candidates do not differ much with the spectrum found in other AUD patients. Broadly two groups can be identified, *i.e.*, a group low at risk for a negative AUD course and a group with higher risk. Up to now, the latter group tends to be screened-out as candidates for LT. However, it remains an open (ethical) discussion whether a higher risk justifies exclusion of a life saving procedure or whether it indicates that higher intensity addiction treatment should be associated within the whole of the treatment trajectory.

Throughout all this process, there is an essential role for an addiction specialist's (team), both in delivering assessment and treatment interventions and as co-ordinators, liaison with specialized addiction care centers. The choice to implement a strong addiction specialists team within the hepatology/transplant center does obviously has major financial implications and in many countries funding this remains extremely challenging.

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P- Reviewer: Grassi A **S- Editor**: Song XX
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i17.2100

World J Hepatol 2015 August 18; 7(17): 2100-2109
ISSN 1948-5182 (online)

New treatment strategies for hepatitis C infection

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Author contributions: Ermis F and Senocak Tasci E contributed equally to this work, generated the tables and figures and wrote the manuscript.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

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Received: April 5, 2015
Peer-review started: April 7, 2015
First decision: May 18, 2015
Revised: June 4, 2015
Accepted: July 18, 2015
Article in press: July 22, 2015
Published online: August 18, 2015

Abstract

Hepatitis C infection can lead to cirrhosis and hepatocellular carcinoma and it is an important cause of mortality and morbidity. Achieving a sustained virological response has been the major aim for decades. Interferon treatment was the primarily developed therapy against the infection. Addition of the guanosine analog ribavirin to stop viral RNA synthesis increased the response rates as well as the adverse effects of the treatment. The increasing demands for alternative regimens led to the development of direct-acting antivirals (DAAs). The approval of sofosbuvir and simeprevir signaled a new era of antiviral treatment for hepatitis C infection. Although the majority of studies have been performed with DAAs in combination with interferon and resulted in a decrease in treatment duration and increase in response rates, the response rates achieved with interferon-free regimens provided hope for patients ineligible for therapy with interferon. Most DAA studies are in phase Ⅱ leading to phase Ⅲ. In the near future more DAAs are expected to be approved. The main disadvantage of the therapy remains the cost of the drugs. Here, we focus on new treatment strategies for hepatitis C infection as well as agents targeting hepatitis C virus replication that are in clinical development.

Key words: Direct-acting antivirals; Eradication; Genotype; Hepatitis C virus infection; Interferon-free; Treatment

Core tip: In this review, we focused on different treatment regimens for hepatitis C infection, especially those including the newly developed and approved direct-acting antivirals. The guidelines are constantly changing in light of new studies. The recommendations of the guidelines are reviewed and consider different genotypes of the virus in addition to the results of ongoing studies. Continuing medical need for agents that act on novel hepatitis C virus targets has resulted in new compounds targeting viral proteins, which is also highlighted in the manuscript.

Ermis F, Senocak Tasci E. New treatment strategies for hepatitis C infection. *World J Hepatol* 2015; 7(17): 2100-2109 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2100.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2100

INTRODUCTION

The hepatitis C virus (HCV), discovered in 1989, affects approximately 3% of the world population, corresponding to 170 million individuals worldwide, and accounts for 500000 deaths per year[1]. Seventy-five percent of the infected patients develop chronic HCV infection, of whom 20% develop cirrhosis[2]. Hepatocellular carcinoma, liver transplantation, and an increase in cardiovascular mortality and morbidity are other outcomes of HCV infection[3]. Eradication of HCV by antiviral treatment can prevent histological deterioration and improve liver histology, along with a decrease in liver-related mortality and morbidity[4]. Pegylated-interferon (Peg-IFN) with ribavirin (RBV) was the standard therapy for hepatitis C until 2011, but new regimens are evolving at a breathtaking pace. In 2011, the first generation protease inhibitors, boceprevir and telaprevir, were approved[5]. In recent years, the Peg-IFN plus RBV regimens gave way to IFN-based strategies combining direct-acting antivirals (DAAs) with Peg-IFN and RBV. Eventually, as an understanding of the HCV life cycle increases, IFN-free combinations of DAAs have evolved to affect all steps of the HCV life cycle and cure most chronically infected patients[6]. The studies suggest use of first generation protease inhibitors (PIs), boceprevir (BOC) and telaprevir (TVR) in the treatment of patients with cirrhosis[7]. Together with the development of DAAs, treatment regimens are characterized by shorter duration, simplified dosing, improved safety profile and effectivity, with > 90% sustained virological response (SVR). Here, new treatment strategies for HCV infection, which aim to eliminate IFN and RBV from the treatment regimen in order to reduce the adverse effects of therapy, are summarized.

TREATMENT REGIMENS FOR CHRONIC HCV GENOTYPE 1 INFECTION

The purpose of HCV therapy is to eradicate the virus. The SVR, indicated by aviremia 24 wk after completion of antiviral therapy for chronic HCV infection, is used to indicate the success of therapy. For years, the standard therapy for HCV infection had been Peg-IFN and RBV for 48 wk independent of the genotype. BOC and TVR were the first NS3/4A PIs targeting NS3 (serine protease) and its cofactor NS4A to block proteolytic maturation of a large portion of the nonstructural region of the HCV polyprotein. After their approval, a combination of Peg-IFN plus RBV plus BOC/TVR began to be used, since monotherapy with BOC or TVR results in the selection of drug resistant variants. The possibility of viral resistance, even in triple combination, resulted in development of DAAs, newer second- and third-generation NS3/4A PIs, NS5B polymerase inhibitors [nucleoside inhibitors (NI) and non-NI (NNIs)], NS5A inhibitors, and inhibitors targeting cyclophilin which is the host factor with an important role in HCV RNA replication[8,9]. These DAAs target specific nonstructural proteins of the virus resulting in disruption of viral replication and infection. NS5B is a RNA-dependent RNA polymerase, essential for viral replication, while NS5A has a role in the organization and regulation of replication[10]. The DAAs in medical use and in development are listed in Table 1[8].

Sofosbuvir was the first NS5B polymerase inhibitor approved for the treatment of chronic hepatitis C by the United States Food and Drug Administration (FDA) in December 2013 and by the European Medical Agency in January 2014. It is well tolerated. The most commonly reported side effects were fatigue, headache, nausea, insomnia, and anemia in the clinical trials performed with sofosbuvir and RBV[11]. Sofosbuvir is administered orally as a 400 mg tablet daily with or without food. Monotherapy is not recommended. Its advantages over previous DAAs are a limited drug-drug interaction profile [inhibits P-glycoprotein transporter so it is not recommended with rifampin, rifabutin, rifapentine, hypericin (a component of St John's Wort), carbamazepine, phenytoin, phenobarbital, oxcarbazepine, tipranovir/ritonavir[12]], and a lack of significant viral resistance. While no dose adjustment is needed in hepatic impairment; the drug is not recommended in end-stage renal disease.

The NEUTRINO study was a phase Ⅲ clinical trial where 327 treatment-naïve patients with HCV genotype 1, 4, 5 or 6 infection received Peg-IFN, RBV, and sofosbuvir for 12 wk, and SVR rates of 89% in genotype 1 were achieved[13]. Also, genotype 1a patients had greater SVR than patients with HCV genotype 1b (92% *vs* 82%, respectively). The SVR at 12 wk was 80% in cirrhotic patients. When the SVR of 82% with 12-wk therapy in genotype 1b subtypes is considered, it is an improvement compared to the first-generation PIs which achieved only 70% SVR with 48 wk of Peg-IFN, RBV, and BOC therapy[14]. The adverse events during the study were associated with Peg-IFN-α and RBV. The randomized phase Ⅱ ATOMIC study compared different schedules of sofosbuvir plus Peg-IFN and RBV in HCV genotype 1 treatment-naïve patients and evaluated the shortest treatment duration. The results showed that sofosbuvir plus Peg-IFN and RBV for a total of 12 wk yielded an SVR rate of 89%, equal to the SVR rate in the extended treatment regimens[15].

Simeprevir is the first available second-generation-NS3/4A- PI which also has an increased efficacy against genotype 1 HCV. The FDA approved simeprevir use in combination with Peg-IFN and RBV in December 2013. It is orally administered as a 150 mg capsule daily with food. As the drug is eliminated by the liver, its use is not recommended in patients with moderate or severe hepatic impairment[16]. The adverse effects reported with simeprevir use are photosensitivity, rash, pruritus, and nausea, which are infrequent[17]. Simeprevir is oxidatively metabolized by the CYP3A subfamily, so drugs that are significant inducers or inhibitors of CYP3A4 are expected to alter the concentration of simeprevir[18]. Because of

Table 1 Direct-acting antivirals (clinical development status in parenthesis)

NS3/4A Protease inhibitors	NS5A inhibitors	Polymerase inhibitors	
		NIs	NNIs
Telaprevir (approved)	Daclatasvir (approved)	Sofosbuvir (approved)	Dasabuvir (phase 3)
Boceprevir (approved)	Ledipasvir (phase 3)	Mericitabine (phase 2)	BMS-791325 (phase 3)
Simeprevir (approved)	Ombitasvir (approved)	VX-135 (phase 2)	PPI-383 (phase 1)
Asunaprevir (phase 3)	GS-5816 (phase 2)		GS-9669 (phase 2)
Danoprevir (phase 3)	ACH-2928 (phase 1)		TMC647055I (phase 2)
Paritaprevir (approved)	ACH-3102 (phase 2)		VX-222 (phase 2)
Vaniprevir (phase 3)	PPI-668 (phase 2)		
Sovaprevir (phase 2)	PPI-461 (phase 1)		
MK-5172 (phase 3)	GSK2336805 (phase 2)		
ACH-2684 (phase 2)	Samatasvir (phase 2)		
Narlaprevir (phase 2)	MK-8742 (phase 3)		
Vedroprevir (phase 2)	BMS-824393 (phase 2)		

NI: Nucleoside inhibitors; NNIs: Non-nucleoside inhibitors.

overlapping resistance, it should not be given to patients with treatment failure for the first-generation PIs, BOC and TVR, nor to genotype 1a patients with the Q80K variant present at baseline, since they had lower SVR rates in the trials[12].

QUEST 1 was a randomized, double blind, placebo-controlled phase III study assessing the efficacy of simeprevir in combination with Peg-IFN and RBV[17]. Treatment-naïve genotype 1 patients randomly received simeprevir plus Peg-IFN and RBV for 12 wk and an additional 12 wk of Peg-IFN and RBV or placebo for 12 wk plus 24 wk of Peg-IFN and RBV. The overall SVR in the simeprevir and placebo group was 80% and 50%, respectively. The subanalysis showed an SVR of 71% for genotype 1a and 90% for genotype 1b patients. The baseline Q80K polymorphism present in 41% of the patients with genotype 1a was associated with lower SVR rates. In the QUEST-2 trial, similar to QUEST-1, an SVR of 81.3% was achieved in the simeprevir-treated group compared with 50% in the placebo group[19], and 91% of patients were suitable for response-guided therapy within the treatment group. The DRAGON study in Japan, assessing the efficacy of simeprevir in treatment-naïve noncirrhotic genotype 1b patients, showed an SVR of 92% in the group treated with simeprevir 100 mg/d plus Peg-IFN and RBV for 24 wk[20]. In the PILLAR phase Ⅱb study, patients received different doses of simeprevir and the highest SVR of 86.1% was achieved in the group receiving simeprevir 150 mg/d plus Peg-IFN for 24 wk[21].

Daclatasvir, the first NS5A inhibitor suppressing HCV RNA synthesis, is a once-daily administered agent approved in Japan, and awaiting FDA approval. In a study by Suzuki *et al*[22], the efficacy and safety of daclatasvir in combination with Peg-IFN and RBV were assessed in treatment-naïve genotype 1 patients where patients receiving daclatasvir 60 mg and Peg-IFN and RBV for 24 or 48 wk showed SVR rates of 90%. There are ongoing studies on daclatasvir in combination with other PIs or NNIs. As these are phase Ⅱ studies, daclatasvir is not expected to be approved by the FDA soon. However, promising results were obtained in a study of daclatasvir in combination with sofosbuvir for 12 wk, which achieved a 98% SVR in 126 treatment-naïve genotype 1 patients[23].

In the treatment of HCV genotype 1 infection, the subtypes are important as patients with genotype 1a tend to have higher relapse rates than patients with genotype 1b with certain regimens. Based on different studies, Miller *et al*[24] recommended 12 wk of IFN-α-2a or b, with RBV and sofosbuvir, or alternatively 12 wk of simeprevir plus 24 wk of Peg-IFN-α-2a or 2b and RBV, or faldaprevir 120 mg for 12 wk plus Peg-IFN-α-2a and RBV for 24 wk for HCV genotype 1-naïve patients in the 2014 United Kingdom consensus guidelines. From June 2014, the company ceased the development of the investigative HCV drug faldaprevir as there was no longer an unmet medical need for the faldaprevir IFN-based regimen[8]. The American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) Recommendations for HCV genotype 1 infection include a combination of weekly Peg-IFN-α, daily weight-based RBV (1000 or 1200 mg in patients < 75 kg or ⩾ 75 kg, respectively), and daily sofosbuvir (400 mg), or a combination of Peg-IFN-α, daily weight-based RBV and daily simeprevir (150 mg) for 12 wk with an additional 12 wk of Peg-IFN and RBV in treatment-naïve and prior relapse patients, and for an additional 36 wk in prior partial and null responders[25,26]. For patients infected with HCV genotype 1b, a combination of weekly Peg-IFN-α, daily weight-based RBV, and daily daclatasvir (60 mg) for 12 wk and an additional 12 wk of Peg-IFN and RBV is recommended. They stated that daclatasvir should be continued in combination with Peg-IFN-α and RBV for an additional 12 wk in patients who did not achieve a HCV RNA level of < 25 IU/mL at week 4 and an undetectable level at week 10[25].

In the near future, IFN-free regimens are expected to replace IFN-based regimens for both non-responders and previously untreated patients. The patients ineligible for therapy with IFN are primary candidates for DAA therapy. Recent EASL recommendations stated that patients with HCV genotype 1 could be

treated with a combination of daily weight-based RBV or daily sofosbuvir (400 mg) for 24 wk as well as daily sofosbuvir (400 mg) and simeprevir (150 mg) for 12 wk. Although adding RBV to this regimen did not show any major advantage, it should be considered in prior non-responder and cirrhotic patients. Daily sofosbuvir (400 mg) and daclatasvir (60 mg) for 12 wk in treatment-naïve patients or for 24 wk in treatment-experienced patients is another IFN-free treatment option recommended by EASL[25]. In addition to the EASL recommendations, a fixed-dose combination of ledipasvir (90 mg/d) and sofosbuvir (400 mg/d) for 12 wk is recommended by AASLD[26].

A sofosbuvir plus RBV combination for 12 wk was evaluated in the ELECTRON trial, achieving SVR rates of 84% in treatment-naïve patients but only 10% in treatment-experienced patients[27]. The QUANTUM study evaluated treatment duration and reported SVR12 and SVR24 rates of 47% and 53%, respectively[28]. The efficacy of simeprevir plus sofosbuvir with or without RBV in prior null responders was assessed in the COSMOS trial for either 12 or 24 wk, and SVR rates of 96.3% with RBV and 92.9% without RBV were achieved[29]. The ION trial evaluated the ledipasvir and sofosbuvir combination[30,31]. Ledipasvir is a NS5A inhibitor, available as part of a fixed-dose combination with sofosbuvir: 90 mg ledipasvir and 400 mg sofosbuvir. Absorption of ledipasvir may decrease with increased gastric pH levels. ION-1 assessed the length of the treatment and SVR was achieved in 97% of patients. In the ION-3 trial treatment length was shortened to 8 wk and an SVR rate of 94% was achieved[32]. In a study by Afhdal *et al*[30], 865 patients were enrolled and divided into 4 groups, and SVR rates for the group treated with ledipasvir and sofosbuvir for 12 wk was 99%, for the group treated with ledipasvir, sofosbuvir and RBV for 12 wk was 97%, for the group treated with ledipasvir and sofosbuvir for 24 wk was 98%, and for the group treated with ledipasvir, sofosbuvir and RBV for 24 wk was 99%[30,33]. Another study by Kohli *et al*[34] tried to reduce treatment time for hepatitis C and assigned 60 patients into 3 groups of 20. High cure rates were achieved for HCV infection with 2 different 3-drug regimens that were given for 6 wk: sofosbuvir, ledipasvir and GS-9451 or GS-9669. The LEAQUE-1 phase Ⅱ study evaluated simeprevir plus daclatasvir 30 mg ± RBV for 12 wk in HCV genotype 1b treatment-naïve patients and prior non-responders[35]. Response rates were 75%-85% and 65%-95%, respectively. Another oral combination recommended by the AASLD and evaluated in SAPPHIRE-I is a combination of RBV-boosted paritaprevir (150 mg), an inhibitor of HCV NS3/4A, ombitasvir (25 mg), twice-daily dasabuvir (referred as 3-D combination) and weight-based RBV for 12 wk, which achieved an SVR of 96% in treatment-naïve genotype 1 infection[33,36].

Patients with prior treatment failure and non-responder patients are still the most difficult group awaiting treatment. In the phase Ⅱ COSMOS trial, 400 mg sofosbuvir and 150 mg simeprevir were given to non-responders or previously untreated patients, and 92%-96% of patients responded to treatment without additional need of RBV[37]. SVR rates did not improve with longer treatment duration or addition of RBV even in the presence of Q80K baseline drug-resistant variants. Daclatasvir is also recommended in treatment-experienced patients. Although mutations associated with resistance to daclatasvir occur at several positions (mutation sites are M28T, Q30E/H/R, L31M/V, P32L, and Y93C/H/N for HCV genotype 1a, and L31F/V, P32L and Y93H/N for HCV genotype 1b), its viral resistance profile does not overlap with that of other DAAs[38,39]. This makes it a good candidate to suppress emerging resistance when combined with other DAAs. The efficacy of daclatasvir 60 mg in combination with sofosbuvir 400 mg with and without RBV for 24 wk was assessed in an open label phase Ⅱ study in patients who failed treatment with TVR or BOC plus Peg-IFN and RBV. The SVR rates were 100% and 98%, respectively, with and without RBV[23]. Although HCV genotype 1a is associated with lower SVR rates, there was no difference between HCV genotype 1a and 1b in this study. The ASPIRE trial evaluated 7 different schedules of simeprevir (100 mg or 150 mg) in combination with Peg-IFN and RBV for the treatment of 452 treatment-experienced patients (16%-20% with cirrhosis)[40]. The SVR rates were 77%-89% in the relapse group and 38%-59% in the non-responder group. The phase Ⅲ PROMISE trial patients (260 treatment-experienced) received simeprevir (150 mg) plus Peg-IFN and RBV for 12 wk followed by Peg-IFN and RBV alone for 12 or 36 wk based on response-guided therapy criteria. An SVR of 79% was achieved and most patients were able to shorten therapy to 24 wk[41]. The ledipasvir and sofosbuvir combination is another treatment option for treatment-experienced patients with HCV genotype 1. Three phase Ⅲ studies were performed in patients who did not respond to IFN therapy with or without a PI[42]; 12 wk of the ledipasvir and sofosbuvir combination ± RBV was given to patients without cirrhosis while 24 wk of therapy was given to patients with cirrhosis. SVR rates were 94% and 96% with and without RBV, respectively. Twenty four wk of therapy was recommended for patients with decompensated cirrhosis while equal efficacy was gained with both 12 and 24 wk of therapy in patients with compensated cirrhosis (96% and 97%, respectively).

Overall, the AASLD-recommended therapy options for treatment-experienced patients are; ledipasvir plus sofosbuvir combination, sofosbuvir plus simeprevir combination with or without RBV, or the triple combination of direct-acting antivirals (3-D) with or without RBV, based on the existence of cirrhosis[42]. The SAPPHIRE-Ⅱ trial included non-cirrhotic patients who failed treatment with Peg-IFN/RBV. The patients received a 3-D combination plus RBV for 12 wk and achieved 96.3% SVR[39]. The PEARL-Ⅱ study evaluated 12 wk of the 3-D regimen with and without RBV in treatment-experienced HCV genotype 1b patients. All patients

in the RBV-containing group and 96% of patients in the RBV-free group achieved an SVR with 12 wk of treatment. Adverse effects were tolerable and fewer in the RBV-free group[43].

TREATMENT REGIMENS FOR CHRONIC HCV GENOTYPE 2 INFECTION

Peg-IFN and RBV had been the standard care of therapy for chronic HCV genotype 2. The duration of treatment was 24 wk and 85%-90% of the patients achieved SVR[44]. With the introduction of DAAs, daclatasvir has been given with Peg-IFN and RBV for 12 wk to treatment-naive HCV genotype 2 patients and 83% of the patients achieved SVR. Since most patients are ineligible, intolerant or unwilling for Peg-IFN, 12 wk of sofosbuvir and RBV combination was found to be highly effective on patients with HCV genotype 2 and was recommended by AASLD and EASL. The FISSION study compared 12 wk of sofosbuvir and RBV with 24 wk of Peg-IFN and RBV where sofosbuvir was found to be superior in the included treatment-naive patient group[12]. 20%-21% of the 499 patients had cirrhosis and the SVR12 rate was 91% for cirrhotic patients with genotype 2 patients and 34% for cirrhotic patients with genotype 3. The FUSION study on the other hand evaluated the treatment duration on treatment-experienced patients of HCV genotype 2. Patients received sofosbuvir plus weight-based RBV for 12 or 16 wk. The SVR rates were 86% and 93%, respectively. 35% of the patients had cirrhosis and the SVR rates were 78% *vs* 60% for genotype 2 (16 wk *vs* 12 wk of treatment) and 61% and 19% for genotype 3 cirrhotic patients, respectively[45]. Although there is no clear benefit shown with 16 wk of treatment, guidelines offer extending treatment to 16 wk in the presence of cirrhosis. The VALENCE study assessed treatment-naive and treatment-experienced patients receiving sofosbuvir and RBV and reported an overall SVR of 93%; 97% (29/30) in treatment-naive noncirrhotic individuals, 100% (2/2) in treatment-naive cirrhotics, 91% (30/33) in treatment-experienced noncirrhotics, and 88% (7/8) in treatment-experienced cirrhotics[46]. The POSITRON study is another phase Ⅲ study involving treatment-experienced patients or patients ineligible for IFN. The efficacy of sofosbuvir and RBV for 12 wk was assessed and the noncirrhotic and cirrhotic patients with genotype 2 achieved 92% and 94% SVR rates, respectively[45]. Based on these studies AASLD and recent EASL guidelines recommend daily sofosbuvir and weight-based RBV for 12 wk for patients with HCV genotype 2 infection in whom prior Peg-IFN and RBV treatment has failed[25,43]. Alternative regimen is a combination of sofosbuvir with Peg-IFN and RBV for 12 wk and it was studied in LONESTAR-2 phase Ⅲ trial where 50% of patients had compensated cirrhosis. Unexpectedly, the results were similar to IFN-free regimen with SVR rates close to 100%[47]. Considering the adverse effects of IFN, sofosbuvir regimen seems like the highly effective and well-tolerated regimen for patients with genotype 2[12].

TREATMENT REGIMENS FOR CHRONIC HCV GENOTYPE 3 INFECTION

Until the development of DAAs, HCV genotype 2 and 3 infections were accepted as easy to treat. Today, with IFN-free regimens, patients with HCV genotype 3 are the most difficult to treat[12]. The recommended regimen for treatment-naïve patients with HCV genotype 3 infection is daily sofosbuvir and weight-based RBV for 24 wk, and was studied in the VALENCE phase Ⅲ trial. SVR rates of 94% for treatment-naïve noncirrhotic patients, 92% for treatment-naïve cirrhotics, 77% for treatment-experienced noncirrhotics, and 60% for treatment-experienced cirrhotics were achieved[48]. The FISSION, FUSION and POSITRON studies assessed the effectiveness of sofosbuvir and RBV in patients with HCV genotype 2 and 3 for 12 and 16 wk, and concluded that previous exposure to Peg-IFN and RBV and disease severity were significant factors in patients with HCV genotype 3 infection[49]. The results were better in treatment-naïve patients and also better with 16 wk of therapy. Taking the VALENCE trial into account, extension of therapy duration as well as addition of another anti-HCV drug should be considered in order to improve the effectiveness of the therapy. Sofosbuvir and daclatasvir combination for 12 wk in treatment-naive or 24 wk in treatment-experienced patients is recommended by EASL guidelines based on the ALLY-3 study, in which 91% treatment-naïve and 86% treatment-experienced patients achieved an SVR with 12 wk of sofsobuvir and daclatasvir combination[50]. Adding RBV is recommended in patients with predictors of poor response to anti-HCV therapy, especially prior non-responders and/or patients with cirrhosis[25]. An alternative regimen for patients with HCV genotype 3 infection in whom prior Peg-IFN and RBV treatment has failed, is retreatment with daily sofosbuvir, RBV and weekly Peg-IFN for 12 wk. In the LONESTAR-2 study, a sofosbuvir, Peg-IFN, and RBV combination resulted in an SVR in 83% of patients infected with genotype 3. The presence of cirrhosis did not affect the response, so genotype 3 treatment-experienced patients with cirrhosis may need IFN-based regimens for a better response[33].

TREATMENT REGIMENS FOR CHRONIC HCV GENOTYPE 4-6 INFECTION

Forty-eight weeks of Peg-IFN and RBV had been the mainstay therapy for patients with genotype 4 infection until the development of DAAs. Recent AASLD and EASLD guidelines recommend the FDA-approved sofosbuvir, Peg-IFN, and RBV regimen for 12 wk[12,25,42]. The genotype 4 cohort of the NEUTRINO study evaluating the efficacy of 12 wk of sofosbuvir and Peg-IFN plus RBV combination achieved a 96% SVR[13]. In a

study conducted in subjects of Egyptian ancestry, 32 treatment-experienced patients were treated with sofosbuvir and RBV for 12 or 24 wk and 87% SVR was achieved in the 24 wk group suggesting this therapy as an effective choice of treatment, especially for patients ineligible for IFN[39]. Simeprevir is effective against HCV genotype 4 infection and the ongoing phase 3 trials including treatment-naïve and treatment-experienced patients have promising results[25]. Therefore, sofosbuvir and simeprevir for 12 wk is an acceptable choice of treatment for patients with HCV genotype 4 infection.

In the PEARL-I study, a 3-D regimen plus RBV was evaluated in 49 treatment-experienced patients without cirrhosis for 12 wk, and 100% of the patients achieved an SVR in the intention-to-treat analysis with no serious adverse events reported[51]. There are not enough studies of DAAs in genotype 4 infection, but the ledipasvir plus sofosbuvir combination for 12 wk is also accepted as effective and recommended by the AASLD based on the SYNERGY trial of 20 patients with HCV genotype 4 where 40% of patients were treatment-experienced and 40% had advanced fibrosis. The overall SVR achieved was 95%[52].

The clinical trials including genotypes 5 and 6 infection are inadequate; in fact no phase Ⅲ data have been presented in treatment-experienced or cirrhotic patients. The only study evaluating treatment-naïve patients with genotype 5 and 6 was the NEUTRINO study where only one patient with genotype 5 and 6 patients with genotype 6 were enrolled[13]. SVR rates achieved for both genotypes were 100% with 12 wk of sofosbuvir and the Peg-IFN and RBV combination. As a result, this combination is the recommended regimen by the AASLD and EASL guidelines.

In addition, ledipasvir is known to have *in vitro* activity against HCV genotype 6 so the ledipasvir plus sofobusvir combination was evaluated in a small, 2-center, open 2 - label study in 25 treatment-naïve and treatment-experienced patients, of whom 2 had cirrhosis. The SVR rate was 96%[53]. This daily fixed-dose combination of ledipasvir and sofosbuvir for 12 wk is a recommended regimen for patients with HCV genotype 6 in whom prior therapy has failed[42].

TREATMENT REGIMENS FOR PATIENTS WITH HUMAN IMMUNODEFICIENCY VIRUS/HCV CO-INFECTION

HCV infection is one of the important causes of comorbidity in patients with human immunodeficiency virus (HIV). Since liver-related mortality became the second highest cause of death in HIV-positive patients, HCV eradication has become obligatory. BOC and TVR were already approved in HCV/HIV co-infection, but since 2013 both sofosbuvir and simeprevir were approved by the FDA to be used in combination with Peg-IFN and RBV to treat patients co-infected with HIV/HCV genotype 1, and sofosbuvir was approved to be used in combination with RBV to treat patients with HIV/HCV genotype 2 and 3[54]. Sofosbuvir plus ledipasvir combination is another alternative for co-infected patients[53]. The limitation of DAAs is pharmacokinetic interactions with antiretroviral drugs. In particular, efavirenz, etravirine, and nevirapine are not recommended with daclatasvir, simeprevir, or sofosbuvir. In addition, daclatasvir dose adjustment is needed in case of ritonavir-boosted atazanavir use[45].

In a study done in Puerto Rico, 12 wk of sofosbuvir plus the Peg-IFN and RBV combination achieved an SVR in 91% of patients[55]. Furthermore, 12 wk of simeprevir plus the Peg-IFN and RBV combination was investigated in HIV/HCV co-infected patients and resulted in a 74% SVR rate in patients with HCV genotype 1[56]. Ledipasvir plus sofosbuvir combination with or without RBV was administered to 12 HIV/HCV genotype 1 patients in a small trial and showed a 100% SVR12 rate[57]. The only study evaluating IFN-free treatment on HIV/HCV co-infected patients is the PHOTON study where sofosbuvir plus RBV was administered to HIV/HCV genotype 1, 2, and 3 patients for 24, 12, and 12 wk, respectively[58]. SVR was achieved in 76% of the patients with genotype 1, 88% of the patients with genotype 2, and 67% of the patients with genotype 3. Studies on treatment regimens including other DAAs are still in progress.

TREATMENT STRATEGIES IN DEVELOPMENT

Commonly used DAAs targeting viral proteins NS3, NS4A, NS5A, and NS5B are mentioned above. One other least characterized viral protein essential for viral replication is NS4B, a 27-kDa integral membrane protein[59]. Several compounds targeting HCV NS4B in antiviral treatment have been mentioned in recent studies. Chen *et al*[60] identified several new azaindole sulfonamides targeting HCV NS4B, and 5-substituted 7-azaindole sulfonamides had the most potent activity with a favorable liver to plasma ratio and excellent oral exposure in rats. Also, NS4B was found to be essential for NS5A phosphorylation[61]. Domain I of NS5A and the C-terminal domain of NS4B were found to be the major determinants mediating the NS5A-NS4B interaction in a study by David *et al*[62]. They suggested that modulation of this interaction could be added to the list of potential NS5A DAA targets.

Another target of antiviral therapy is the ion channel activity of HCV p7. The p7 channel is crucial for virus replication *in vitro*, playing a role in virus assembly and release[63]. BIT225 is a novel small molecule identified as an inhibitor of the p7 ion channel that completed 2 phase I human trials. Phase IIa studies are still ongoing. Luscombe *et al*[64] reported the inhibitory effect of BIT225 as well as its strong synergy with Peg-IFN and RBV, which makes it a good candidate for use in combination therapy. Although the mechanism of action is not fully understood, amantadine and rimantadine

are known to inhibit the HCV p7 ion channel[65]. In the p7 channel, there are 6 equivalent hydrophobic pockets and nearby there are 3 aromatic amino acids (His17, Phe20, and Trp21). Du *et al*[66] focused on the nuclear magnetic resonance structure of HCV p7 and found that the best binding site of amantadine was Trp21. The binding sites and interactions mentioned in their study may help the future development of p7 channel inhibitors. Clinical data is only available for amantadine[67] while other compounds are reported to inhibit the HCV p7 ion channel, including long alkylchain iminosugars and hexamethylene amiloride[68,69].

HCV genomic RNA holds genetic information for viral proteins and contains regions of sequences required for HCV replication or translation. Antisense oligonucleotides (ASOs) have been identified in order to inhibit HCV RNA replication and viral polyprotein synthesis *in vitro*. Studies on HCV-infected patients show that modified ASOs can result in decrease in viral load of > 2 log units[70]. A new generation of ASOs, locked nucleic acids (LNA), show improved affinity of binding to RNA targets, increased sequence specificity, and lower toxicity[71]. An internal ribosome entry site (IRES) is a nucleotide sequence that allows translation initiation in the middle of a messenger RNA (mRNA) sequence in HCV[72]. Host microRNA (miR-122) plays a role in HCV replication *in vitro* and is joined directly to a region in the IRES[73]. Studies in primates demonstrated that LNA-based ASOs targeting miR-122 can be delivered to the liver for 12 wk with no adverse effects and result in a virological response of > 2 log units in plasma HCV RNA levels, and decreased expression of cellular mRNA carrying the miR-122 region[74,75]. In a study by Laxton *et al*[76], 47 ASOs were screened and 7 hits with selectivity index higher than 10 were identified; 5 hits targeting NS5a and 2 hits targeting IRES (seq132 and seq207-250a). Seq132 ASO showed potent antiviral activity (95% to 98% antiviral activity) with low cytotoxicity. The possible antiviral mechanisms of seq132 were highlighted as antagonism of miR122 binding, loss of HCV sequences due to RNase H activity, and local destabilization of the IRES secondary structure. In addition, Bhat *et al*[77] revealed the interaction between ribosomal protein S5 (RPS5) and HCV IRES. They found that blocking RPS5 in 40S ribosome subunits results in inhibition of HCV IRES activity. Therefore, HCV translation is inhibited. This may help in designing potential peptide mimics as potential antiviral molecules.

CONCLUSION

Development of DAAs represents significant progress in the treatment of HCV infection. IFN-based regimens cause adverse events, which make tolerance and compliance an important issue. Therefore, the new era of IFN-free regimens is highly accepted in the treatment of treatment-naïve and treatment-experienced patients, especially in individuals in whom IFN is absolutely contraindicated. Current IFN-free regimens offer SVR rates above 90% and 12-wk treatment duration in both groups. Recently, sofosbuvir, found to be effective against all genotypes, and simeprevir, daclatasvir, and ledipasvir are the most promising DAAs. The once daily dosing, low pill burden, pan-genotypic activity, lower rate of drug-drug interactions, fewer side effects, and shorter treatment duration makes these regimens more tolerable.

The most important issue of DAA treatment is the cost and availability. The regimens are extremely expensive so the cost should be reduced to provide universal access in all patients with HCV, especially in developing countries. In these regions, as for HIV treatment, International Health Organizations may help with free drug distribution and treatment follow-up.

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P- Reviewer: Colacino JM **S- Editor**: Ma YJ
L- Editor: Cant MR **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i17.2110

World J Hepatol 2015 August 18; 7(17): 2110-2118
ISSN 1948-5182 (online)

MINIREVIEWS

Recent advances in mouse models of obesity- and nonalcoholic steatohepatitis-associated hepatocarcinogenesis

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Author contributions: Nakagawa H solely contributed to this manuscript.

Supported by Grants from the Japanese Society of Gastroenterology; The Tokyo Society of Medical Sciences; and Kanae Foundation for the Promotion of Medical Science.

Conflict-of-interest statement: The author has no conflicts of interest regarding this study.

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Received: April 7, 2015
Peer-review started: April 9, 2015
First decision: May 14, 2015
Revised: May 28, 2015
Accepted: June 30, 2015
Article in press: July 2, 2015
Published online: August 18, 2015

Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer, and obesity has been established as a risk factor for HCC development. Nonalcoholic steatohepatitis (NASH) is apparently the key link between obesity and hepatocarcinogenesis, and obesity also accelerates HCC development synergistically with other risk factors, such as hepatitis virus infection and alcohol consumption. As an explanation for the pathogenesis of NASH, the so-called "two-hit" theory has been widely accepted, but recently, a better model, the so-called "multiple-hits hypothesis" was proposed, which states that many disease-promoting factors may occur in parallel, rather than consecutively. However, the overall mechanism remains largely unknown. Various cell-cell and organ-organ interactions are involved in the pathogenesis of NASH, and thus appropriate *in vivo* disease models are essential for a deeper understanding. However, replicating the full spectrum of human NASH has been difficult, as NASH involves obesity, insulin resistance, steatohepatitis, fibrosis, and ultimately HCC, and the lack of an appropriate mouse model has been a considerable barrier to determining the missing links among obesity, NASH, and HCC. In recent years, several innovative mouse models presenting obesity- and NASH-associated HCC have been established by modified diets, chemotoxic agents, genetic manipulation, or a combination of these factors, shedding some light on this complex network and providing new therapeutic strategies. Thus, in this paper, I review the mouse models of obesity- and NASH-associated HCC, especially focusing on recent advances and their clinical relevance.

Key words: Obesity; Metabolic syndrome; Nonalcoholic steatohepatitis; Hepatocellular carcinoma; Mouse model

Core tip: Obesity is a recognized risk factor for the development of hepatocellular carcinoma (HCC) and nonalcoholic steatohepatitis (NASH), which in turn can

trigger hepatocarcinogenesis. Once, no appropriate mouse model allowed exploration of the associations among obesity, NASH, and HCC, but several innovative mouse models have become established in recent years. These models have afforded new insights into the mechanisms of disease and have suggested new therapeutic strategies. Therefore, this paper reviews mouse models of obesity- and NASH-associated HCC, focusing on recent advances and clinical relevance thereof.

Nakagawa H. Recent advances in mouse models of obesity- and nonalcoholic steatohepatitis-associated hepatocarcinogenesis. *World J Hepatol* 2015; 7(17): 2110-2118 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2110.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2110

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and a leading cause of cancer-related death[1]. Although the short-term prognosis of patients with HCC has improved due to advances in early diagnosis and treatment, the long-term prognosis remains unsatisfactory, with a low overall survival of 22%-35% at 10 years[2,3]. More than 90% of HCC develops in the context of chronic liver damage and inflammation[4], and obesity has recently been established as a risk factor for HCC development, with a 1.5-4-fold increased risk[5,6]. Accumulating evidence indicates that nonalcoholic fatty liver disease (NAFLD) is the key link between obesity and hepatocarcinogenesis, with evidence indicating that obesity accelerates HCC development synergistically with other risk factors, such as chronic viral hepatitis and alcohol consumption[7,8]. Because the prevalence of obesity has been increasing worldwide, its possible association with hepatocarcinogenesis has attracted considerable attention in recent years.

NAFLD, the most common chronic liver disease in developed countries, has been recognized as a hepatic manifestation of metabolic syndrome. NAFLD encompasses a wide range of pathological conditions, ranging from "simple" steatosis to the more aggressive form "nonalcoholic steatohepatitis (NASH)," which is accompanied by inflammation, cell death, and scarring (fibrosis) that eventually results in cirrhosis and/or HCC. Histologically, NASH is characterized by the presence of ballooning hepatocytes and lobular inflammation with or without perisinusoidal fibrosis in addition to steatosis[9]. To explain the pathogenesis of NASH, the so-called "two-hit" theory proposed by Day *et al*[10] in 1998 has been widely accepted. They suggested that after a first hit (hepatic steatosis), another hit is needed for NASH to develop. Since then, various factors such as pro-inflammatory cytokines, dysregulation of adipokines, gut-derived endotoxins, oxidative stress, endoplasmic reticulum (ER) stress, lipotoxicity, altered gut microbiota, and activation of intracellular signaling pathways have been suggested to be the second hit, and which factor(s) is (are) the true driving force of disease progression from simple steatosis to NASH has been debated[11-15]. Recently, a better model, the so-called "multiple-hits hypothesis" was proposed by Tilg *et al*[16], which states that many of the events described above may take place in parallel, rather than consecutively. However, the overall mechanism is very complex and remains largely unknown. Thus, no specific established treatment exists to prevent NASH progression and subsequent HCC development.

A good mouse model is indispensable for understanding such a complicated liver disease, involving interactions with various other organs, such as the gut, brain, and adipose tissue, because mice are readily amenable to genetic modifications and easy to handle. Although various NASH mouse models have been reported, most of the existing models do not replicate the full spectrum of human NASH, which includes obesity, insulin resistance, steatohepatitis, fibrosis, and ultimately HCC[17]. In particular, mimicking hepatocarcinogenesis is difficult, and the lack of appropriate mouse model(s) has been a considerable barrier for understanding the underlying pathogenesis behind the link(s) among obesity, NASH, and HCC.

Several innovative mouse models presenting obesity- and NASH-associated HCC have been established in recent years using modified diets, chemotoxic agents, genetic manipulation, or a combination of these factors. They have provided new insights into mechanisms as to how obesity and NASH promote HCC and have also resulted in the suggestion of new therapeutic strategies. Although several review articles on mouse models of NASH have been recently published[17-21], to our knowledge, no review focusing on mouse models of obesity- and NASH-associated hepatocarcinogenesis has been published. Thus, in this paper, I review the mouse models of obesity- and NASH-associated HCC, especially focusing on recent advances and their clinical relevance.

MOUSE MODELS OF OBESITY- AND NASH-ASSOCIATED HCC

Obesity- and NASH-associated HCC mouse models have been created using modified diets, chemotoxic agents, genetic manipulation, or a combination of these elements. Here, we classify current mouse models into four groups and discuss their characteristics: dietary models, diet in combination with chemotoxic agent models, genetically engineered models, and genetic manipulation in combination with dietary models (Table 1).

DIETARY MODELS

Long-term high-fat diet

A high-fat diet (HFD) is widely used to cause obesity and hepatic steatosis in mice, and long-term feeding

Table 1 Mouse models of obesity- and nonalcoholic steatohepatitis - associated hepatocellular carcinoma

	Obesity	Insulin resistance	Steatosis	Steatohepatitis	Fibrosis	HCC
Dietary models						
Long-term HFD	Yes	Yes	Yes	Yes	Yes	Yes
CD-HFD	Yes	Yes	Yes	Yes	Yes	Yes
High fat and fructose diet	Yes	Yes	Yes	Yes	Yes	Yes
Dietary in combination with chemotoxic agent models						
DEN with HFD	Yes	Yes	Yes	No	No	Yes
STZ with HFD	No	Yes	Yes	Yes	Yes	Yes
DMBA with HFD	Yes	N/A	Yes	No	No	Yes
Genetically engineered models						
Liver-specific PTEN knockout mice and p110α transgenic mice	No	No	Yes	Yes	Yes	Yes
Liver-specific NEMO knockout mice	No	No	Yes	Yes	Yes	Yes
miR-122 knockout mice	No	Yes	Yes	Yes	Yes	Yes
FXR knockout mice	No	Yes	Yes (mild)	Yes (mild)	Yes	Yes
AOX knockout mice	No	N/A	Yes	Yes	No	Yes
MAT1A knockout mice	No	No	Yes	Yes	Yes	Yes
FLS mice crossed with *ob/ob* mice	Yes	Yes	Yes	Yes	Yes	Yes
Dominant negative form of RARα transgenic mice	No	No	Yes	Yes	No	Yes
Genetic manipulation in combination with dietary models						
MUP-uPA transgenic mice with HFD	Yes	Yes	Yes	Yes	Yes	Yes
Adiponectin knockout mice with HFD	Yes	Yes	Yes	Yes	Yes	No (adenoma)
AIM knockout mice with HFD	Yes	Yes	Yes	No	No	Yes
MC4R knockout mice with HFD	Yes	Yes	Yes	Yes	Yes	Yes

N/A: Not assessed; HCC: Hepatocellular carcinoma; HFD: High-fat diet; CD: Choline-deficient; DEN: Diethylnitrosamine; STZ: Streptozotocin; DMBA: Dimethylbenz(a)anthracene; NEMO: Nuclear factor κB essential modulator; miR: MicroRNA; FXR: Farnesoid X receptor; AOX: Acetyl CoA oxidase; MAT1A: Methionine adenosyl transferase 1A; FLS: Fatty liver Shionogi; RAR: Retinoid acid receptor; AIM: Apoptosis inhibitor of macrophage; MC4R: Melanocortin 4 receptor.

of HFD also induces insulin resistance. Although HFD-induced fatty liver has been considered to represent "simple" steatosis, some recent studies have shown that an extended period of HFD feeding (*e.g.*, 60 wk) in C57/BL6J mice could induce steatohepatitis with weak perisinusoidal fibrosis, and also neoplastic lesions, including HCC, in approximately 50% of mice[22,23], which suggests that excess intake of dietary fat can be a causal factor in HCC development. However, the phenotypes induced by HFD are variable according to mouse strains, fat content in the diet, and the composition of the dietary fat. Importantly, disruption of IRS-1, a mediator of insulin and IGF signals in C57/BL6J mice, was found to dramatically protect against long-term HFD-induced liver tumorigenesis despite the presence of severe insulin resistance and marked postprandial hyperglycemia[23]. This finding suggests that hyperglycemia itself may not play a role in NASH or NASH-associated hepatocarcinogenesis.

Choline-deficient high-fat diet

A methionine- and choline-deficient (MCD) diet is a classic and widely adopted model for studying NASH. Because methionine and choline are essential for hepatic β-oxidation and the production of very low-density lipoprotein (VLDL), their deficiency leads to extensive hepatic lipid accumulation, and steatohepatitis subsequently develops, which resembles the pathology of human NASH[17]. However, the MCD diet does not cause obesity, insulin resistance, or metabolic syndrome; rather, it induces weight loss and even cachexia. To overcome these limitations, Wolf *et al*[24] combined choline deficiency with an HFD on the basis of clinical observations of choline deficiency in patients with NASH. CD-HFD-fed C57/BL6 mice revealed obesity and insulin resistance as well as a human NASH-like pathology, with mild pericellular fibrosis. Furthermore, long-term feeding (12 mo) resulted in spontaneous HCC development in 25% of mice, including classical trabecular HCC. In contrast, only 2.5% of HFD-fed mice developed a liver tumor over the same time period. In this model, hypernutrition and choline deficiency activated intrahepatic natural killer T (NKT) cells, which in turn enhanced hepatocyte lipid uptake and aggravated liver steatosis *via* secretion of LIGHT [a member of the tumor necrosis factor (TNF) superfamily]. Also, $CD8^+$ T cells, NKT cells, and associated inflammatory cytokines cooperatively cause liver damage and nuclear factor κB (NF-κB) activation, which facilitates the NASH-to-HCC transition. Thus, hepatocyte-lymphocyte cross talk may be a promising therapeutic target for NASH and NASH-associated HCC.

High-fat and fructose diet

Recently, long-term feeding (12 mo) of an HFD in combination with fructose syrup has also been reported to cause the development of liver tumors, including HCC, as well as steatohepatitis and mild fibrosis[25]. This model exemplifies the clinical setting, the so-called "American lifestyle-induced obesity syndrome." However, the incidence of macroscopically visible nodules was not very high, and characterization of the tumors and

analysis of the mechanism were not sufficient due to the small number of occurrences. Further studies are needed with this promising mouse model.

DIET IN COMBINATION WITH CHEMOTOXIC AGENT MODELS

Diethylnitrosamine with HFD

Diethylnitrosamine (DEN) is the most commonly used genotoxic chemical carcinogen to develop HCC because inducing HCC is easy, and DEN-induced HCC shows histology and gene expression similar to human HCC, especially a poor prognosis[4]. A single intraperitoneal injection of DEN to 2-wk-old male mice is sufficient to induce HCC[26,27], and HFD feeding to DEN-injected mice strongly enhances HCC development[28]. The greatest benefit of this model is that HCC is easy to induce and its incidence rate is almost 100% at 8 mo of age. However, the initiation step of HCC development basically depends on artificial, toxic DNA damage, and non-tumor liver tissue corresponds to simple steatosis, lacking inflammatory cell infiltration and fibrosis. However, this model is suitable for analyzing obesity-associated promotion and progression processes in HCC. In fact, HFD feeding resulted in systemic low-grade inflammation, and ablation of interleukin-6 (IL-6) and the TNF receptor 1 abrogated their tumor-promoting effects, suggesting that IL-6 and TNFα play important roles in the promotion of obesity-associated HCC[28].

Streptozotocin with HFD

Streptozotocin (STZ), a drug particularly toxic to β-cells in the pancreas, is widely used to induce diabetes in mice[29]. Recently, STZ in combination with an HFD has been reported to induce NASH and spontaneous HCC development[30]. In this model, low-dose STZ was injected subcutaneously at 2 d after birth, and then HFD feeding was started at 4 wk of age. Steatohepatitis occurred at 8 wk of age along with pericellular fibrosis, and all male mice developed well-differentiated-type HCC at 20 wk. These findings lead to a revolutionary hypothesis that insufficient insulin signaling, rather than hyperinsulinemia, plays a key role in NASH-associated HCC. The advantage of the STZ with HFD model is that it can replicate human NASH-like pathology and can also induce spontaneous HCC in a relatively short time. However, these mice do not show obesity or insulin resistance. In addition, whether the cancer initiation process depends on NASH-induced chronic inflammation or STZ administration remains unclear because STZ is known as a carcinogen, and administration of STZ alone can induce HCC in Syrian golden hamsters[31].

Dimethylbenz(a)anthracene with HFD

Dimethylbenz(a)anthracene (DMBA), a chemical carcinogen that causes an oncogenic Ras mutation, is widely used to induce skin and breast cancer[32]. Recently, Yoshimoto *et al*[33] reported that neonatal treatment with DMBA on the dorsal surface at day 4-5 followed by an HFD for 30 wk induced HCC in all male mice, whereas none of the normal diet-fed DMBA-treated mice developed HCC. This paper elegantly showed that increased deoxycholic acid (DCA) by an obesity-induced alteration in the gut microbiota provoked a senescence-associated secretory phenotype (SASP) in hepatic stellate cells (HSCs), which in turn secreted various inflammatory and tumor-promoting factors, eventually resulting in the malignant transformation of initiated hepatocytes by DMBA. In addition, lowering the DCA concentration by treatment with ursodeoxycholic acid (UDCA) or antibiotics inhibited HCC development significantly. Thus, although UDCA failed to improve the histology in patients with NASH compared with placebo in some clinical trials[34,35], a possibility exists that long-term treatment with UDCA may prevent HCC development in obese patients with NASH, independent of NASH disease status. Also, the gut microbiota may be a future therapeutic target candidate. However, DMBA treatment can induce oncogenic mutations in various cell types. In fact, lung cancer also developed with DMBA treatment regardless of diet in this study. Thus, although they showed the absence of the hot spot mutation of the H-Ras gene in HSCs, whether SASP of HSCs is specific to this model or a universal phenomenon using other obesity- and NASH-associated HCC models should be examined.

GENETICALLY ENGINEERED MODELS

Liver-specific PTEN knockout mice and p110α transgenic mice

A tumor suppressor PTEN negatively regulates the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathways by its lipid phosphatase activity. Liver-specific PTEN knockout mice generated by crossing PTEN flox/flox mice with Albumin-Cre transgenic mice spontaneously develop steatohepatitis with marked triglyceride accumulation *via* the activation of Akt signaling and upregulation of PPARγ and SREBP1[36]. Additionally, all mice developed hepatocellular adenomas, and 66% developed HCC by 74-78 wk of age. This mouse model is one of the most well-known genetically engineered models of NASH-associated HCC. Hepatocyte-specific transgenic mice of p110α, which is a catalytic subunit of PI3K, also showed similar phenotypes[37]. Although pathological features of these mice are similar to human NASH-associated HCC, these models do not show obesity or metabolic syndrome, but instead are hypersensitive to insulin.

Liver-specific NF-κB essential modulator knockout mice

NF-κB essential modulator (NEMO), also known as inhibitor of NF-κB subunit γ (IKKγ), controls NF-κB activation through its interaction with ubiquitin chains[4]. Liver-specific NEMO knockout mice exhibit spontaneous liver damage, hepatosteatosis, fibrosis, and HCC

development[38]. Although the mechanism as to how NEMO deletion causes liver steatosis remains unclear, death receptor-mediated and oxidative stress-dependent hepatocyte death are triggers of liver damage and inflammation. The disease process in this model is similar to human HCC, which is a consequence of chronic inflammation, hepatocyte death, and compensatory proliferation. However, because this model does not show obesity or metabolic syndrome, it is not suitable for studying the metabolic consequences of NAFLD.

MicroRNA-122 knockout mice

MicroRNA(miR)-122 is a predominant liver microRNA, accounting for 70% of the total miRNAs in the liver. Both mice with germline knockout and liver-specific knockout of miR-122 revealed steatohepatitis and fibrosis, and also developed HCC, including metastasizing aggressive cases, at 12-17 mo of age[39,40]. Enhanced lipogenesis and suppressed lipid secretion through loss of miR-122 cause liver steatosis in these mice. Loss of miR-122 promotes HCC development not only indirectly through chronic inflammation, but also directly through induction of the epithelial-mesenchymal transition (EMT) by E-cadherin downregulation[40], which plays an important role in EMT in HCC[41]. These findings are pathophysiologically important and clinically relevant because the expression of miR-122 is significantly decreased in patients with NASH[42]. Furthermore, adeno-associated virus-mediated delivery of miR-122 suppressed Myc-driven HCC[39], suggesting the potential utility of miR122 delivery for patients with HCC.

Farnesoid X receptor knockout mice

The nuclear bile acid receptor farnesoid X receptor (FXR) is highly expressed in the liver and intestine, and cross talk between these two organs plays a key role in maintaining bile acid homeostasis[43]. FXR also plays important roles in lipid and glucose metabolism and regulation of insulin sensitivity by regulating the expression of various metabolic genes. FXR knockout mice exhibit chronic liver damage with mild steatosis and fibrosis, and aged mice develop spontaneous HCC by the age of 12-16 mo[44]. Although steatosis in FXR knockout mice is mild, the combination of LDL receptor knockout and HFD induces significant steatosis and ballooning degeneration of hepatocytes[45]. Currently, FXR is an attractive therapeutic target in the clinical setting because the FXR agonist obeticholic acid was found to show significant improvements in histological features of NASH in a recent multicenter placebo-controlled randomized trial[46]. However, treatment with obeticholic acid was associated with some disadvantageous effects, such as increases in serum cholesterol and low-density lipoprotein cholesterol concentrations, a decrease in serum high-density lipoprotein cholesterol concentrations, and increased insulin resistance. Furthermore, recent experimental studies using tissue-specific FXR knockout mice indicated complicated cross talk between liver and intestine FXR, and even an apparently opposite function of FXR signaling for NASH progression between the liver and intestine[47-49]. Further studies are needed to clarify the roles of this signaling process in NASH and NASH-associated HCC.

Other genetically engineered models

Acetyl CoA oxidase (AOX) is the rate-limiting enzyme of the peroxisomal β-oxidation of long-chain fatty acids. AOX knockout mice have defective peroxisomal β-oxidation and exhibit steatohepatitis without fibrosis[50]. AOX knockout mice also exhibit hepatocellular adenoma and HCC by 15 mo of age. However, hepatic steatosis is reversed by a compensatory increase in fatty acid oxidation by 6-7 mo of age.

Methionine adenosyltransferase 1A (MAT1A) is the rate-limiting enzyme of methionine metabolism in the liver. MAT1A knockout mice develop spontaneous steatohepatitis and HCC *via* abnormal expression of genes involved in lipid and carbohydrate metabolism[51]. However, MAT1A knockout mice do not show obesity or metabolic syndrome, except hyperglycemia.

The "fatty liver Shionogi" (FLS) mouse strain shows lipid deposition in hepatocytes from the neonatal stage, and the degree of hepatic lipid accumulation increases as the mouse grows without obesity. Additionally, crossing FLS mice with leptin mutant *ob/ob* mice induces obesity, metabolic syndrome, NASH, and spontaneous HCC development[52]. Although the mechanism of this phenotype in FLS mice is not fully understood, it is believed to be caused by a complex polygenic trait.

Transgenic mice expressing the dominant-negative form of the retinoid acid receptor (RAR)α in hepatocytes display microvesicular steatosis and spotty necrosis at 4 mo of age, and aged mice develop spontaneous HCC at the age of 12-18 mo[53]. In dominant-negative RARα transgenic mice, mitochondrial β-oxidation of fatty acids is decreased, but peroxisomal β-oxidation and microsomal ω-oxidation are increased, resulting in an enhanced accumulation of reactive oxygen species.

GENETIC MANIPULATION IN COMBINATION WITH DIETARY MODELS

Major urinary protein-urokinase-type plasminogen activator transgenic mice with HFD

Major urinary protein (MUP)-urokinase-type plasminogen activator (uPA) mice are uPA transgenic mice under the control of the mature hepatocyte-specific promoter for MUP[54]. MUP-uPA mice express the uPA protein in mature hepatocytes, where it accumulates in the ER, leading to chronic ER stress in the hepatocytes. We recently reported that feeding an HFD to *MUP-uPA* mice resulted in steatohepatitis that closely resembles the pathology of human NASH, with ballooning degeneration, hepatocyte death, and pericellular/bridging fibrosis, eventually leading to spontaneous development of HCC, including classic trabecular HCC and steatohepatitic HCC, by 40 wk of

age[55]. In this mouse model, the vicious cycle of ER stress and hypernutrition synergistically aggravates lipid accumulation in the liver *via* sterol regulatory element binding protein (SREBP) activation, which leads to excess oxidative stress, ballooning degeneration, and susceptibility to lipotoxic cell death. In parallel, increased TNFα expression during this process further accelerates NASH and HCC development in a TNF receptor 1-IKK-NF-κB-dependent manner. Reducing ER stress using chemical chaperones significantly improved liver pathology, suggesting that interrupting this vicious cycle might be a promising therapeutic target for NASH and HCC.

Isolated premalignant HCC progenitor cells (HcPC) from DEN-injected mice can be transplanted into MUP-uPA mice[56], and the NASH-like microenvironment created by HFD feeding significantly promotes the progression from HcPC to HCC[55]. This approach may allow us to separate cell-autonomous effects of genetic manipulation as well as dietary conditions within pre-neoplastic cells from effects exerted within the surrounding liver parenchyma, and furthermore, allow us to separate the effects of NASH on the tumor progression process from the tumor initiation process. In this regard, this is a unique mouse model to analyze the mechanisms of NASH-associated HCC.

Adiponectin knockout mice with HFD

Adiponectin, one of the major adipokines, possesses anti-inflammatory and insulin-sensitizing properties, and levels typically decline with increasing body weight[57]. Hypoadiponectinemia is seen in patients with NASH, and reduced adiponectin levels are associated with more extensive liver steatosis and necroinflammation[58]. Adiponectin knockout mice have insulin resistance and glucose intolerance on a normal diet[59]. HFD-fed adiponectin knockout mice exhibit NASH-like pathology, including hepatocyte ballooning, spotty necrosis, and pericellular fibrosis *via* increased hepatic expression of TNFα and SREBP1c at 24 wk, and furthermore, some HFD-fed adiponectin knockout mice (12.5%) develop hepatocellular adenoma at 48 wk[60]. This experimental study showed that too little adiponectin can be a causal factor of obesity-associated liver tumorigenesis. However, several recent epidemiological studies have shown that a higher serum adiponectin level is associated with an increased risk of future HCC development[61-63]. The effects of too much adiponectin on hepatocarcinogenesis remains poorly understood, and some reports have shown cancer-promoting effects of adiponectin[64,65]. Because adiponectin signaling is considered to be a promising target of NASH, further basic and clinical studies should be conducted.

Apoptosis inhibitor of macrophage knockout mice with HFD

Circulating apoptosis inhibitor of macrophage (AIM) is incorporated into adipocytes and hepatocytes, and inactivates cytoplasmic fatty acid synthase *via* direct binding. Thus, AIM knockout mice show increased steatosis and lipid accumulation in the liver after HFD feeding. Furthermore, all HFD-fed AIM knockout mice spontaneously develop HCC without apparent liver inflammation or fibrosis by 55 wk of age[66]. AIM accumulates on the HCC cell surface and activates the complement cascade, provoking HCC cell necrosis. Administration of recombinant AIM was found to prevent HCC development in HFD-fed AIM knockout mice. These findings suggest that delivery of AIM to HCC cells may be a novel therapeutic strategy against obesity-driven HCC.

Melanocortin 4 receptor knockout mice with HFD

Melanocortin 4 receptor (MC4R) is expressed in the hypothalamic nuclei and has been implicated in the regulation of food intake and body weight. Several pathogenic mutations in the *MC4R* gene have been reported, especially in early-onset obesity[67]. MC4R knockout mice in combination with an HFD exhibit steatohepatitis, which is associated with obesity, insulin resistance, and dyslipidemia. In addition, all HFD-fed MC4R knockout mice developed HCC after 1 year of HFD feeding[68]. Although the detailed mechanism remains unclear, it seems likely that the hepatic phenotype in MC4R knockout mice results from loss of its function in the brain because the expression of MC4R mRNA is basically restricted to the brain.

CONCLUSION

Table 1 lists the key features of each mouse model described in this review. The dietary models most closely mimic the human condition. However, HCC development is slow and its incidence relatively low. The combination of DEN and HFD affords a model superior in terms of certainty and ease of use. The STZ/HFD combination triggers HCC development relatively quickly. The MUP-uPA/HFD model is a unique in that the effects of NASH on tumor progression can be separated from the effects on tumor initiation. Although the histopathological characteristics of miR-122- and FXR-knockout mouse livers are not identical to those of human NASH patients (weak steatosis and lack of ballooning hepatocytes), the phenotypes of mice so affected are important pathophysiologically and clinically relevant, as discussed above. It is important to understand the advantages and disadvantages of each mouse model and to choose the model that is optimal for the experimental purpose.

The tumor-promoting effects of obesity and NASH are caused by not only changes in the hepatic microenvironment, such as excess lipid accumulation, oxidative stress, ER stress, and inflammatory cytokines secreted by immune cells and fibroblasts, but also by changes in the extrahepatic environment, such as visceral fat accumulation, altered gut microbiota, and hypothalamic appetite dysregulation. Such a complex situation, composed of various cell-cell and organ-organ interactions, cannot be reproduced *in vitro*, and

appropriate *in vivo* disease models are essential to fully understand it. Recent advances in mouse models shed some light on this complicated network and have suggested several new therapeutic targets. However, we are still far from a complete understanding and no specific established treatment exists to prevent NASH or NASH-associated HCC. Thus, further studies and novel strategies clarifying the entire picture of this complex disease are still needed to translate the findings obtained from experimental research into clinical practice.

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P- Reviewer: Gu Y, Kusmic C **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Liu SQ

World Journal of Hepatology

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DOI: 10.4254/wjh.v7.i17.2119

World J Hepatol 2015 August 18; 7(17): 2119-2126
ISSN 1948-5182 (online)

Model for end-stage liver disease-Na score or Maddrey discrimination function index, which score is best?

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Conflict-of-interest statement: Amieva-Balmori M, Mejia-Loza SMI, Ramos-González R, López-Luria A, García-Ruiz E, Pérez y López N, Juárez-Valdés EI and Zamarripa-Dorsey F do not have conflict of interest. José María Remes-Troche is member of the Advisory Board for Takeda Pharmaceuticals, Alfa-Wasserman, Almirall and Janssen. Also, he is Speaker for Nycomed-Takeda, Advance Medical, Endomedica, Astra-Zeneca and Bristol-Myers-Squibb.

Data sharing statement: No additional data are available.

Received: October 10, 2014
Peer-review started: October 13, 2014
First decision: December 17, 2014
Revised: June 24, 2015
Accepted: July 16, 2015
Article in press: July 17, 2015
Published online: August 18, 2015

Abstract

AIM: To compare the ability of model for end-stage liver disease (MELD)-Na and Maddrey discrimination function index (DFI) to predict mortality at 30 and 90 d in patients with alcoholic hepatitis (AH).

METHODS: We prospectively assessed 52 patients with AH. Demographic, clinical and laboratory parameters were obtained. MELD-Na and Maddrey DFI were calculated on admission. Short-term mortality was assessed at 30 and 90 d. Receiver operating characteristic curve analysis was performed.

RESULTS: Thirty-day and 90-d mortality was 44% and 58%, respectively. In the univariate analysis, sodium levels was associated with mortality at 30 and 90 d (P = 0.001 and P = 0.03). Child stage, encephalopathy, ascites, or types of treatment were not associated with mortality. MELD-Na was the only predictive factor for mortality at 90 d. For 30-d mortality area

under the curve (AUC) was 0.763 (95%CI: 0.63-0.89) for Maddrey DFI and 0.784 for MELD-Na (95%CI: 0.65-0.91, P = 0.82). For 90-d mortality AUC was 0.685 (95%CI: 0.54-0.83) for Maddrey DFI and 0.8710 for MELD-Na (95%CI: 0.76-0.97, P = 0.041).

CONCLUSION: AH is associated with high short-term mortality. Our results show that MELD-Na is a more valuable model than DFI to predict short-term mortality.

Key words: Alcoholic hepatitis; Model for end-stage liver disease-Na; Maddrey; Mortality

Core tip: Alcoholic hepatitis (AH) is a severe condition associated with high mortality. The model for end-stage disease (MELD) score is widely used to predict mortality in end-stage liver disease, and the addition of sodium (MELD-Na) increase its utility. However, few studies have evaluated the utility of MELD-Na in AH. In this study, we found that MELD-Na is useful for predicting 90-d mortality in patients with AH and preserve prognostic advantage over Maddrey discrimination function index score. It represents a valuable tool to stratify patients by risk, however further studies are required to validate the prognostic utility of MELD-Na score in patients with AH.

Amieva-Balmori M, Mejia-Loza SMI, Ramos-González R, Zamarripa-Dorsey F, García-Ruiz E, Pérez y López N, Juárez-Valdés EI, López-Luria A, Remes-Troche JM. Model for end-stage liver disease-Na score or Maddrey discrimination function index, which score is best? *World J Hepatol* 2015; 7(17): 2119-2126 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i17/2119.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i17.2119

INTRODUCTION

It is estimated that 6% of the Mexican population is dependent on alcohol which equals to 4.9 million people[1]. Chronic alcohol consumption is the leading cause of liver failure in our country, and alcohol intake > 120 g/d is a factor associated with the development of alcoholic hepatitis (AH)[1-3]. AH was first described by Gordon Beckett in 1961 and clinical description of the syndrome is still valid after 50 years[4]. This entity is an acute form of alcohol induced liver injury that is seen in patients who consume large quantities of alcohol during a prolonged period of time. Its spectrum is wide and ranges from a silent disease to fulminant liver failure with a high mortality rate. Patients with severe AH have been reported to have 30-d mortality up to 50%[5,6].

Therefore, assessment of the disease severity becomes an important and practical issue for clinicians involved in the management of patients with AH[6]. There are several prognostic models to assess severity in patients with AH including the Maddrey's discriminant function index (DFI)[7], the Glasgow AH score (GAHS)[8], the age- bilirubin- international normalized ratio (INR) - creatinine (ABIC) score[9], the Lille score[10] and the model for end-stage liver disease (MELD)[11]. Among the many scoring systems, the DFI is the most used. A score higher than 32 in the DFI is considered as a severe AH and mortality rates are close to 65% at 28 d[8,12]. Also, DFI allows identifying patients who may benefit from treatment with steroids[13]. However, some studies have shown that the cut-point of 32 of DFI could be inaccurate and higher cut-offs have been proposed (from 37 to 44)[12,14].

Although MELD score was designed for evaluation of patients awaiting liver transplantation[15], its use has been expanded and now, is used as a prognostic scale in various liver diseases such as AH[16,17], viral hepatitis[18], hepatocellular carcinoma[19] and autoimmune diseases[20]. As hyponatremia is associated with poor prognosis in cirrhosis, inclusion of serum sodium (Na) into the MELD (MELD-Na) was found to improve its predictive value in chronic liver diseases[21,22]. MELD-Na is more efficient than MELD to identify subjects with poor outcome and significantly increase the efficacy of the score to predict waitlist mortality[22].

Several studies have examined the use of MELD in assessing the severity of AH[12,16,17,23,24] and sensitivity and specificity in predicting 30-d mortality ranges from 75% to 86%. Few studies, have evaluated the usefulness of the MELD-Na in AH[24-26] and results are controversial.

As sodium abnormalities are close related to end stage liver disease conditions such as ascites and hepatorenal syndrome (HRS), we hypothesize that MELD-Na is better to predict short-term mortality in patients with AH compared to the Maddrey DFI (the most used score).

MATERIALS AND METHODS

Patients and procedures

We prospectively identified 52 patients admitted to our Gastroenterology Service (Hospital Juarez de Mexico, Mexico City, Mexico) between March 2011 and March 2013, with a diagnosis of AH and history of long alcohol consumption. The patients were diagnosed with AH based on the following clinical and biochemical characteristics: excessive alcohol consumption (> 100 g/d) at least 2 mo prior to admission, serum total bilirubin level above 5 mg/dL, aspartate/alanine aminotransferase ratio above 2, aspartate aminotransferase level below 300 IU/mL, history of longstanding alcoholism, and finally the absence of a coexistent primary cause of liver disease, such as viral hepatitis, drug induce liver diseases, non-alcoholic hepatitis, autoimmune hepatitis and hepatocellular carcinoma. Only patients with laboratory values available within 24 h of admission were included.

Data collection

The following data were obtained for all patients: age, sex, history of alcohol consumption, clinical complications at admission and during hospitalization [ascites, hepatic encephalopathy, renal failure (as defined as serum creatinine $\geqslant$ 1.5 mg/dL), HRS, bacterial infections and gastrointestinal bleeding]; length of hospital stay, treatment received and cause of death. The analytical parameters at admission or within 48 h of admission included serum glucose, cholesterol, triglycerides, sodium, albumin, aminotransferases, bilirubin and creatinine levels, blood urea nitrogen, INR, leukocyte count, neutrophil count, platelet count, and hematocrit.

Short-term mortality was assessed at 30 and 90 d. The Child-Turcotte-Pugh (CTP) score was calculated for all patients regardless the presence or absence of cirrhosis. Medical treatment was also assessed. Both, Maddrey DFI and MELD-Na scores were based on clinical and laboratory parameters collected at the time of diagnosis of AH. Maddrey DFI was calculated using the formula: DFI = 4.6 × (PTsec-control PTsec) + serum total bilirubin in mg/dL. MELD-Na score was calculated using the formula: 3.8 (log bilirubin mg/dL) + 11.2 (ln INR) + 9.6 (ln creatinine mg/dL) + 6.4 + 1.59 (135 - Na). Maddrey DFI and MELD-Na scores higher than 32 and 21, respectively, were considered as a more severe disease and associated with poor outcomes[6,8]. Patients received oral corticosteroids if they met the following criteria: a modified Maddrey's DFI > 32 or hepatic encephalopathy at admission, recent onset of jaundice, and biochemical changes suggestive of AH. Prednisone was given orally (40 mg/d) for 4 wk followed by a taper of 2-3 wk. Contraindications for corticosteroid treatment were severe bacterial infections, renal dysfunction, diabetes mellitus with poor metabolic control, and the presence of acute gastrointestinal bleeding. For those patients, pentoxifylline was prescribed 400 mg thrice/d.

Statistical analysis

Continuous variables were expressed as means with standard deviation and range. Categorical variables were expressed with percentage. χ^2 analysis was used to compare categorical variables, and continuous variables were analyzed using the Student *t*-test and Mann-Whitney. The primary end point was death from any cause at 30 and 90 d from hospital admission. With the significant prognostic variables obtained from the univariate analysis, multivariate logistic regression was carried out using forward selection model.

The accuracy of the MELD-Na score was compared with the Maddrey DFI score, through the analysis of their area under the receiver operating characteristic (AUROC) curve. Receiver operating characteristic (ROC) curves were generated to assess the prognostic utility of Maddrey DF and MELD score, evaluated by their ability to rank patients according to the risk of mortality at 30 and 90 d. An AUROC value of > 0.70 was considered clinically relevant. Comparison between AUROC curves was performed by the method of Hanley and McNeil[27] using MedCalc version 9.3.0.0. (Medisoftware, Mariakerke, Belgium). From ROC curves coordinates, cut-off points with best sensitivity and specificity of the different scores were determined. A *P* value less than 0.05 was considered statistically significant. Statistical interpretation of data was performed using statistical package for social sciences (SPSS) version 16.0 for Windows (SPSS, Inc., Chicago, Illinois, United States). The Institutional Review Board and the Ethics Committee approved this study.

RESULTS

Fifty two subjects met the inclusion criteria. Forty eight patients (92%) were males, and mean age was 42.8 ± 8.7 years. Mean alcohol consumption per day was 283 g and mean days of continuous alcohol consumption prior to admission was 24 d. Thirty eight patients (73%) developed ascites and 24 (46%) encephalopathy. A concomitant infection process was detected in 16 (31%) of the patients; 7 (44%) had a urinary tract infections and 5 (31%) spontaneous bacterial peritonitis, and 4 (25%) had both urinary tract infection and spontaneous bacterial peritonitis. At admission mean MELD score was 30.8 ± 3.3, MELD-Na was 27.5 ± 7.7 (range, 12 to 48) and Maddrey DFI values was 79.7 ± 54 (range, 13 to 321). Specific treatment for AH was used in 75% (*n* = 39) of patients: pentoxifylline was used in 48% (*n* = 25), prednisone alone was used in 17% (*n* = 9), and 10% (*n* = 5) received prednisone in combination with pentoxyfylline.

Mortality rate at 30 d was 44% (*n* = 23), and the attributable causes were: multiple organ failure in 44% (*n* = 10), renal insufficiency from HRS in 44% (*n* = 10) and gastrointestinal hemorrhage in 13% (*n* = 3). Mortality rate at 90 d was 57.6% (*n* = 30) and multiple organ failure occurred in 47% (*n* = 13), renal insufficiency from HRS in 40% (*n* = 12) and gastrointestinal hemorrhage in 13% (*n* = 5). The variables that were significantly associated with 30-d and 90-d mortality in the univariate analysis are presented in Tables 1 and 2.

Lower sodium levels (*P* = 0.019), higher total bilirubin levels (*P* = 0.018), higher creatinine levels (*P* = 0.001), Child class C (*P* = 0.023), development of HRS (*P* = 0.001) and a higher MELD-Na (*P* = 0.003) were significant factors associated with 30-d mortality. Lower sodium levels (*P* = 0.03), higher total bilirubin levels (*P* = 0.009), higher creatinine levels (*P* = 0.01), higher INR (*P* = 0.002), higher prothrombin time (*P* = 0.0003), lower cholesterol levels (*P* = 0.01), Child class C (*P* = 0.05), development of HRS (*P* = 0.05) and a higher MELD-Na (*P* = 0.01) were significant factors associated with 90-d mortality. Treatment with specific medication, development of infections or gastrointestinal bleeding did not influence survival.

In the multivariate logistic regression, HRS was the strongest and independent predictor of mortality at 30-d (*P* = 0.001). MELD-Na was a predictor of mortality at 90-d (*P* = 0.036) (Table 3). No additional variables

Table 1 Univariate analysis between survived and deceased patients at 30 d

Variables	Survived (n = 29)	Deceased (n = 23)	P
Demographic			
Age (yr)	40 ± 9.6	44 ± 12	0.114
Alcohol consumption per day (g/d)	291 ± 140	302 ± 159	0.809
Male, n (%)	28 (97)	20 (87)	0.222
Laboratory parameters at admission			
White blood cell counts (10^3/μL)	17362 ± 9807	21772 ± 10131	0.11
Glucose (mg/dL)	102 ± 49	102 ± 61	0.987
Sodium (mmol/L)	132 ± 6	128 ± 6	0.019[a]
Total bilirubin (mg/dL)	17.3 ± 8.9	23.6 ± 9.4	0.018[a]
AST (IU/L)	172 ± 111	189 ± 93	0.55
ALT (IU/L)	66.9 ± 40.5	71.5 ± 33	0.66
γGT (IU/L)	369 ± 254	291 ± 183	0.282
Alkaline phosphatase (IU/L)	254 ± 109	222 ± 112	0.344
Creatinine (mg/dL)	1.61 ± 1.5	3.5 ± 2.5	0.001[a]
INR	2.05 ± 0.6	2.49 ± 1.48	0.079
Prothrombin time (s)	23.14 ± 8.1	27.2 ± 11.2	0.142
Albumin (mg/dL)	2.8 ± 0.5	2.5 ± 0.6	0.73
Cholesterol (mg/dL)	150.8 ± 68	116 ± 53	0.081
Triglycerides (mg/dL)	222 ± 122	230 ± 178	0.869
Calcium (mg/dL)	7.9 ± 0.7	7.5 ± 1.1	0.10
Clinical manifestations at admission			
Ascites, n (%)	25 (86)	22 (95)	0.468
Child status			0.023[a]
Grade B, n (%)	6 (20)	0	
Grade C, n (%)	23 (80)	23 (100)	
Encephalophaty, n (%)			0.335
None	13 (45)	7 (30)	
Stage Ⅰ	4 (14)	5 (22)	
Stage Ⅱ	8 (28)	10 (43)	
Stage Ⅲ	4 (14)	1 (4)	
Hepatorenal syndrome, n (%)	5 (17)	14 (61)	0.001[a]
Severity of liver disease at admission			
MELD-Na score	25.5 ± 8	31.9 ± 6	0.003[a]
Maddrey DFI	69.4 ± 42	93 ± 53.8	0.08
MELD	32.1 ± 6.5	25.1 ± 2.9	0.79

[a]P < 0.05 *vs* survived patients at 30 d. MELD: Model for end-stage liver disease; DFI: Discrimination function index; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transpeptidase.

increased the predictive accuracy of MELD-Na (bilirubin, INR, and creatinine, as factors included in Maddrey DF and/or MELD score, were excluded from the analysis).

A clinical utility analysis was performed using the pre-established cut-off values for Maddrey DFI and MELD-NA (> 32 and > 21, respectively) and considering death at 30 and 90 d as the outcome. Sensitivity, specificity, positive predictive values, negative predictive values and accuracy are shown in Table 4. Receiver operating characteristic curves were created in order to estimate the predictive accuracy of the different scores to evaluate 30-d and 90-d mortality (Figure 1). For 30-d mortality the area under the curve (AUC) was 0.763 (95%CI: 0.63-0.89) for Maddrey DFI and 0.784 for MELD-Na (95%CI: 0.65-0.91, P = 0.82). For 90-d mortality the AUC was 0.685 (95%CI: 0.54-0.83) for Maddrey DFI and 0.8710 for MELD-Na (95%CI: 0.76-0.97, P = 0.041).

DISCUSSION

Excessive alcohol consumption is a social problem in Mexico and it has been estimated that alcohol related liver diseases (ALD) are responsible to approximately 9% of all diseases in Mexico[28]. A subset of patients with ALD will develop severe AH (AH), which has a substantially worse short-term prognosis[29]. The true prevalence is unknown, but histologic studies of patients with ALD suggest that AH may be present in as many as 10%-35% of hospitalized alcoholic patients[30,31].

Although a recent publication reported that the inpatient mortality rate in AH has decreased in the United States (from 10.07% in 2002 to 5.76% in 2010), in this cohort of Mexican patients with AH we found a high mortality rate, 44% at 30 d and 57.6% at 90 d[32]. Our results are similar to that reported in a recent multicentric study in Mexico in 175 patients with AH, where overall and 90-d mortality rate were 36% and 51%, respectively[3]. Similar to other cohorts, we found that most common causes of mortality were portal hypertension and HRS. This increased mortality rate could be explained by socioeconomic factors, quality of health services, higher amount of alcohol consumption in Mexican patients, as well as genetic factors[3,29]. For

Table 2 Univariate analysis between survived and deceased patients at 90 d

Variables	Survived (n = 22)	Deceased (n = 30)	P
Demographic			
Age (yr)	41 ± 9	44 ± 11	0.27
Alcohol consumption per day (g/d)	284 ± 148	303 ± 143	0.584
Male, n (%)	21 (95)	27 (90)	0.94
Laboratory parameters at admission			
White blood cell counts (10^3 /μL)	284 ± 148	303 ± 143	0.584
Glucose (mg/dL)	108 ± 59	99 ± 49	0.55
Sodium (mmol/L)	133 ± 5	129 ± 6	0.03[a]
Total bilirubin (mg/dL)	16 ± 8	22 ± 10	0.009[a]
AST (IU/L)	192 ± 137	177 ± 84	0.61
ALT (IU/L)	103 ± 150	67 ± 39	0.24
γGT (IU/L)	577 ± 656	399 ± 480	0.29
Alkaline phosphatase (IU/L)	281 ± 91	211 ± 105	0.01[a]
Creatinine (mg/dL)	2 ± 1.8	3 ± 2.11	0.01[a]
INR	2 ± 0.4	3 ± 1.3	0.002[a]
Prothrombin time (s)	19 ± 4	28 ± 12	0.0003[a]
Albumin (mg/dL)	3 ± 0.5	3 ± 5	0.54
Cholesterol (mg/dL)	176 ± 90	119 ± 51	0.01[a]
Triglycerides (mg/dL)	240 ± 163	226 ± 162	0.76
Calcium (mg/dL)	7.9 ± 0.8	7.3 ± 1.5	0.28
Clinical manifestations at admission			
Ascites, n (%)	19 (86)	28 (93)	0.71
Child status			0.05[a]
Grade B, n (%)	8 (37)	3 (10)	
Grade C, n (%)	14 (63)	27 (90)	
Encephalophaty, n (%)	0 (0)		
None	10 (45)	0 (30)	0.106
Stage Ⅰ	8 (37)	3 (10)	
Stage Ⅱ	4 (18)	19(63)	
Stage Ⅲ		8 (26)	
Hepatorrenal syndrome, n (%)	5 (22)	16 (53)	0.05[a]
Severity of liver disease at admission			
MELD-Na score	24.95 ± 8	30.9 ± 7.79	0.01[a]
Maddrey DFI	68.5 ± 42	88.3 ± 48.6	0.12
MELD	22.1 ± 7.5	23.1 ± 3.1	0.28

[a]P < 0.05 *vs* survived patients at 90 d. MELD: Model for end-stage liver disease; DFI: Discrimination function index; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGT: Gamma glutamyl transpeptidase.

Table 3 Variables with significance in the multivariate logistic regression analysis

	Significance	Odds ratio	95%CI
30-d mortality			
MELD-Na	0.11	1.25	0.78-1.7
Maddrey DFI	0.14	1.14	0.82-3.04
Bilirubin	0.45	0.7	0.47-3.6
Creatinine	0.38	0.31	0.74-1.98
INR	0.41	0.78	0.68-1.52
Hepatorenal syndrome	0.001	11.5	2.7-48.11
90-d mortality			
MELD-Na	0.036	1.19	1.06-1.232
Maddrey DFI	0.09	1.03	0.87-1.86
Bilirubin	0.23	0.67	0.65-3.56
Creatinine	0.35	0.37	0.8-4.2
INR	0.17	0.272	0.78-2.6

MELD: Model for end-stage liver disease; DFI: Discrimination function index; INR: International normalized ratio.

example, several studiesin Mexican-American and Mestizo populations have identified a virtual absence of some of the alcohol "protective" genes variations (*ADH1B* and *ALDH2*) and a high frequency of CPY2E c2polymorphic allele, which result in increased enzymatic activity,augmented acetaldehyde production, and more severe liver damage[33,34].

Many strategies have been used to predict morbidity and mortality in AH allowing a better medical support for those very ill patients. Such strategies include the search for single parameters (*i.e.,* alkaline phosphatase) or the development of scoring systems like the Maddrey DFI, the GAHS, the ABIC, the Lille score and MELD[20-24]. According to our results we propose that MELD-Na is also a useful scoring system to predict severity in AH.

Although several studies have explored the clinical utility of severity scores in AH, results are variable. For example, Lafferty *et al*[35] in a cohort of 182 patients prospectively evaluated GAHS, MELD, ABIC and DFI scores anddid not found differences in the outcome among them. Other studies have focused in the specific use of MELD in evaluating the severity of AH. Dunn *et al*[11] in a study with 73 patients with AH found that a MELD score of 21 had the highest sensitivity and specificity to predict mortality at 30 and 90 d. In

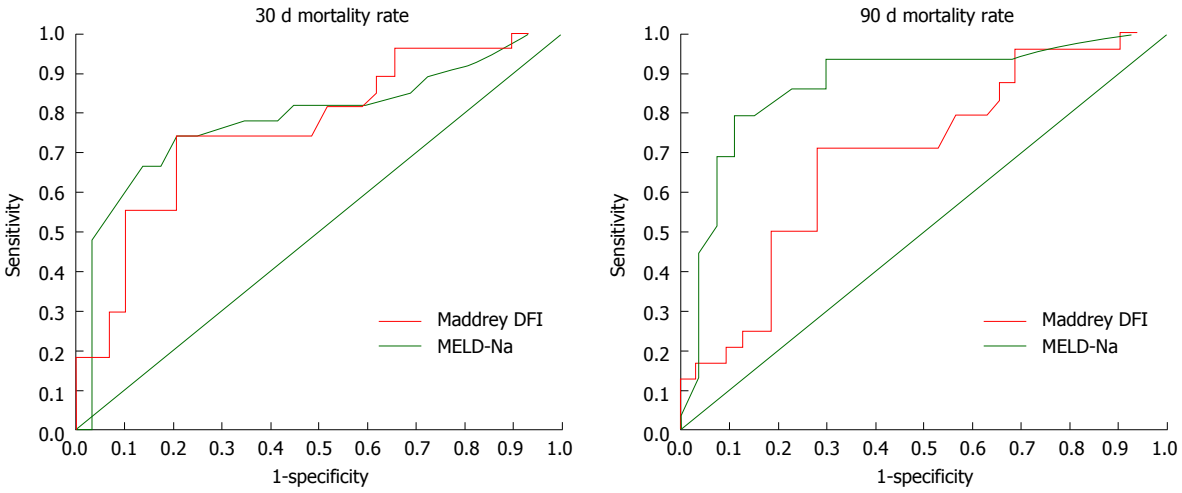

Figure 1 Comparison of Maddrey discrimination function index and model for end-stage liver disease-Na in predicting mortality at 30 and 90 d in alcoholic hepatitis. For 30-d mortality the area under the curve (AUC) was 0.763 for Maddrey DFI and 0.784 for MELD-Na (P = 0.82). For 90-d mortality the AUC was 0.685 for Maddrey DFI and 0.8710 for MELD-Na (P = 0.041). MELD: Model for end-stage liver disease; DFI: Discrimination function index.

Table 4 Clinical utility analysis at 30 and 90 d to predict mortality %

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
Maddrey DFI > 32					
Mortality at day 30	96	21	53	86	57
Mortality at day 90	93	22.7	62.2	71.4	63.5
MELD-Na > 21					
Mortality at day 30	85	31	53	69	57.1
Mortality at day 90	87	40	66	69	67.3

MELD: Model for end-stage liver disease; DFI: Discrimination function index.

contrast, Monsanto *et al*[17] in a small sample size (n = 45) and retrospective study found that Maddrey DFI was a more valuable model to predict short-term mortality in patients with AH. Recently, a prospective study in 47 subjects with AH, found that both the MELD score and the Maddrey DFI score at admission were strong and equally good predictors of 28-d mortality in patients with AH[16]. However, in this study the optimal Maddrey DFI cut off point corresponding to the optimal MELD score was higher than the conventional one and the authors propose that MELD score may be used as an alternative to DFI score for predicting short-term mortality in AH[17].

Three previous studies have compared the ability of MELD-NA to predict mortality compared to other scores[24-26]. The first study, a small sample size study from the Mayo Clinic, showed that MELD-Na was a better predictor of 180-d mortality than MELD in patients with ascites[26]. In another study, Kasztelan-Szczerbinska *et al*[25] compared Maddrey DFI, CPT, GAHS, ABIC MELD and MELD-Na in 116 subjects with AH and no statistically significant differences in the models' performances were found. Specifically for MELD-Na, the AUC was 0.83 to predict mortality at 90 d, similar to our findings. In a more recent study, nine scoring models were compared in 71 biopsy-proven patients with AH and all models showed excellent negative predictive values and MELD modifications incorporating sodium did not confer any prognostic advantage over classical MELD[24]. Interestingly, in this cohort the 30-d mortality and 90-d mortality rates were lower compared to other studies (14.1% and 19.7%, respectively). Also the authors did not report the incidence of ascites and HRS.

Hyponatremia is a common clinical problem in patients with end stage liver disease, and has a close relationship with portal hypertension, ascites and HRS. Low sodium levels are related to the impairment of renal solute-free water excretion most likely due to an increased vasopressin secretion, which results in increased sodium retention and reduced renal free water clearance, which predispose to life threatening conditions in the cirrhotic such as HRS and refractory ascites[36]. Also, hyponatremia represents an independent risk factor for brain edema, a fatal complication of acute liver failure[37,38]. Interestingly, we found that low sodium levels were associated with mortality at 30 and 90 d. Also, HRS was associated to mortality in the univariate and multivariate analysis. Thus, for us, was not surprisingly that MELD-Na had better clinical utility performance and ability to predict mortality at 90 d compared to Maddrey DFI.

We need to acknowledge that although we showed that MELD-Na is a useful tool to predict mortality, the treatment provided to our patients did not influence in their survival. Currently, corticosteroids or pentoxifylline

are the main pharmacological treatment options; though the outcomes from the therapies are poor. Because of the limitations in the therapeutic options, it is no doubt that there is a critical need for the newer and more effective.

Other limitations that should be acknowledge include: a small sample size, some patients with suspected AH could not be included in the final analysis because they had incomplete laboratory parameters at admission, lack of comparison with other models that have been shown utility in Mexican population such as ABIC[3] and histological diagnosis of AH was not confirmed. However, several studies have shown that diagnosis of AH is confirmed in almost 80% of the suspected cases when high levels of recent alcohol consumption is confirmed and histological confirmation is not required[39,40]. Intriguingly, we did not find that encephalopathy, ascites and CPT were associated with mortality. However this finding is probably related with the power in our small sample size. Finally, although we found a better performance for MELD-Na to predict 90 d mortality, the clinical relevance of our findings should be assessed in future prospective, multicentric and larger sample size studies.

In conclusion, AH, is associated with high short-term mortality. We found that MELD-Na is useful for predicting 90-d mortality in patients with AH and preserve prognostic advantage over Maddrey DFI score. It represents a valuable tool to stratify patients by risk, however further studies are required to validate the prognostic utility of admission MELD-Na score in patients with AH.

COMMENTS

Background

Alcoholic hepatitis (AH) is a severe condition associated with high mortality. The model for end-stage disease (MELD) score is widely used to predict mortality in end-stage liver disease, and the addition of sodium (MELD-Na) increase its utility. However, few studies have evaluated the utility of MELD-Na in AH.

Research frontiers

Few studies have compared the ability of MELD-NA to predict mortality compared to other scores.

Innovations and breakthroughs

In this study, the authors found that MELD-Na is useful for predicting 90-d mortality in patients with AH and preserve prognostic advantage over Maddrey discrimination function index (DFI) score.

Applications

MELD-Na may represent a valuable tool to stratify patients by risk and to predict in patients with AH.

Terminology

AH: Alcoholic hepatitis; MELD: Model for end-stage disease; MELD-Na: MELD plus sodium; DFI: Discriminant function index; ALD: Alcoholic liver diseases.

Peer-review

This is a well written small study that recommends the use of MELD-Na in the prognostic scoring of patients with acute hepatitis. It warrants publication in its current form.

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P- Reviewer: Bramhall S, Jin B **S- Editor**: Tian YL
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