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WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Severe alcoholic hepatitis-current concepts, diagnosis and treatment options

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Abstract

Alcoholic hepatitis (AH) is an acute hepatic manifestation occurring from heavy alcohol ingestion. Alcoholic steatohepatitis (ASH) is histologically characterized by steatosis, inflammation, and fibrosis in the liver. Despite the wide range of severity at presentation, those with severe ASH (Maddrey's discriminant function $\geqslant$ 32) typically present with fever, jaundice, and abdominal tenderness. Alcohol abstinence is the cornerstone of therapy for AH and, in the milder forms, is sufficient for clinical recovery. Severe ASH may progress to multi-organ failure including acute kidney injury and infection. Thus, infection and renal failure have a major impact on survival and should be closely monitored in patients with severe ASH. Patients with severe ASH have a reported short-term mortality of up to 40%-50%. Severe ASH at risk of early death should be identified by one of the available prognostic scoring systems before considering specific therapies. Corticosteroids are the mainstay of treatment for severe ASH. When corticosteroids are contraindicated, pentoxifylline may be alternatively used. Responsiveness to steroids should be assessed at day 7 and stopping rules based on Lille score should come into action. Strategically, future studies for patients with severe ASH should focus on suppressing inflammation based on cytokine profiles, balancing hepatocellular death and regeneration, limiting activation of the innate immune response, and maintaining gut mucosal integrity.

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Key words: Alcoholic steatohepatitis; Infection; Renal failure; Corticosteroids; Pentoxifylline

Core tip: We should further explore novel molecular targets to restore altered gut mucosal integrity, suppress inflammation based on cytokine profiles, promote hepatic regeneration, and limit innate immune responses in severe alcoholic steatohepatitis.

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the main causes of end-stage liver disease worldwide[1]. ALD has a broad disease spectrum, encompassing simple steatosis, steatohepatitis, and cirrhosis. In particular, the short-term mortality in patients with severe alcoholic steatohepatitis (ASH) has been extremely high up to 40%-50%[2,3]. Although several therapeutic measures are now available to improve survival in those with severe alcoholic hepatitis (AH), overall prognoses remain gloomy.

Recently, severe AH with a significant morbidity and mortality ranks among the most costly diseases during hospitalization in the United States[4]. Thus, the early

detection of high-risk patients and prompt intervention may assist in the alleviation of healthcare cost associated with severe AH. Accordingly, the accurate prognostic stratification is crucial for individualized therapeutic decisions in patients with AH. Several prognostic scoring systems, to date, have been developed and validated for use in those with AH[5-10].

The clinical syndrome of jaundice and liver function abnormalities in alcohol abusers is generally called AH, which has often been referred to as "acute alcoholic hepatitis" historically. However, despite the sudden onset of the clinical presentation, this term seems to fade into the mists of history now that AH is usually associated with extensive fibrosis or cirrhosis and often follows a protracted natural course.

ASH is a pathologic disease entity, defined as the coexistence of steatosis, hepatocellular ballooning, neutrophilic infiltration, and perisinusoidal fibrosis[11]. ASH is not exclusively accompanied by AH but can be superimposed on any different stages of ALD comprising steatosis, steatohepatitis, fibrosis, and cirrhosis[12,13]. However, it is not much well-known which patients with ASH will progress to clinically evident AH. In addition, the true incidence and prevalence of ASH or AH among alcohol abusers remain unclear due to the uncertainties behind a clinical diagnosis of AH and the limited number of studies with liver biopsy to ascertain a histologic diagnosis of ASH.

Recently, the updated practice guidelines for management of ALD have been released from the European Association for the Study of the Liver[14] as well as the American Association for the Study of Liver Diseases[15]. Herein, we attempt to address some issues regarding different types of alcohol-induced liver failure, new prognostic scoring systems, general therapeutic measures, and potential specific therapies in patients with severe ASH from a clinical perspective.

DIAGNOSIS

Different types of alcohol-induced liver failure

Traditionally, there are two different types of liver failure, which have different prognoses and call for different therapeutic approaches. One is acute liver failure (ALF), which occurs suddenly in patients without previous any liver disease. The other is chronic liver failure (CLF) due to chronic hepatic decompensation (CHD), which is found in those with end-stage liver cirrhosis as a result of slow progression of underlying liver disease. Since the advent of albumin dialysis, a new subtype of CLF, that is, acute-on-chronic liver failure (ACLF) has been widely recognized and highlighted in the field of clinical practice[16-18]. This new entity is characterized by an acute and rapid deterioration within several weeks on the top of underlying compensated liver disease, mostly cirrhosis, leading to deep jaundice, renal impairment, hepatic encephalopathy, and multi-organ failure in the early stage[19]. Indeed, among the patients hospitalized for alcoholic cirrhosis, ACLF showed a 3-mo mortality rate of 60% *vs* that of 20% in case of CHD, illustrating the severity of this new clinical syndrome[20]. Currently, an excessive pro-inflammatory response to bacterial components such as gut microbiota and lipopolysaccharide (LPS) seems to play an important role in ACLF, linking the gut, liver, and portal to systemic circulation[21].

In the same manner, alcohol can also instigate two different types of liver injury including ACLF and CHD. The most critically ill patients with alcohol-induced liver failure are some people suffering from severe ASH, mostly superimposed on alcoholic cirrhosis, and secondly those with decompensated cirrhosis. Precipitating events such as variceal hemorrhage, infection, and hepatitis B viral reactivation are usually crucial for the onset of ACLF, given that the rapid and aggressive control of these triggers can allow a complete reversal of ACLF. In this regard, an early use of transjugular intrahepatic portosystemic shunt effectively prevented the development of ACLF in patients with high-risk varices[22]. Similarly, early suppression of hepatitis B viremia by tenofovir prevented those with spontaneous reactivation of hepatitis B presenting as ACLF from progressing to multi-organ failure[23]. CHD is the most frequent subtype of alcohol-induced liver failure and is characterized by the complications of portal hypertension and mild to moderate jaundice in the early stage. The 1-year mortality rate was 29% in case of the appearance of ascites; however, it was 64% if hepatic encephalopathy occurred as a complication of portal hypertension in patients with alcoholic cirrhosis[24]. ACLF is the less frequent subtype of alcohol-induced liver failure but accounts for more than 40% of emergency hospitalization due to alcoholic cirrhosis in tertiary referral hospitals[20]. ACLF can be induced by several precipitating events in patients with alcoholic cirrhosis; however, one of the most common triggers is severe ASH, which occurs in roughly 25% of the patients with ACLF[20].

Prognostic scoring systems

The best way to reverse alcohol-induced ACLF is to detect and control severe ASH as early as possible, which is less likely to recover spontaneously. In this regard, a variety of prognostic scores have been developed primarily to select patients with severe ASH at high risk of early (1, 2, or 3 mo) death[5-8,10]. There are several disease-specific prognostic models (MDF: Maddrey's Discriminant Function; GAHS: Glasgow Alcoholic Hepatitis Score; ABIC: Age-Bilirubin-INR-Creatinine Score; Lille model; MAGIC: Model for Alcoholic hepatitis to Grade the severity In an Asian patient Cohort,) and a non-disease-specific model (MELD: Model for End-Stage Liver Disease) (Table 1)[5-8,10].

MDF is still one of the most commonly used prognostic models to predict survival outcomes in patients with ASH with 32 of a cutoff value[10,25]. Severe ASH (MDF $\geqslant$ 32) mostly progress to the systemic inflammatory response syndrome (SIRS) and multi-organ failure, which are often seen in other types of ACLF. MAGIC is a recently developed, new model to predict liver-related death in Asian patients hospitalized for AH[8]. The unique

Table 1 Components of clinical scoring systems to assess prognosis in alcoholic hepatitis

	Bilirubin	PT/INR	Cr/BUN	WBC	Age	Albumin	Potassium	Change in bilirubin from day 0 to day 7
MDF[10]	+	+	-	-	-	-	-	-
MELD[6]	+	+	+	-	-	-	-	-
GAHS[7]	+	+	+	+	+	-	-	-
ABIC[5]	+	+	+	-	+	+	-	-
Lille[9]	+	+	+	-	+	+	-	+
MAGIC[8]	+	+	+	-	-	-	+	+

PT/INR: Prothrombin time/international normalized ratio; Cr/BUN: Creatinine/blood urea nitrogen; WBC: White blood cell; MDF: Maddrey's discriminant function; MELD: Model for end-stage liver disease; GAHS: Glasgow alcoholic hepatitis score; ABIC: Age, serum bilirubin, INR, and serum creatinine; MAGIC: Model for alcoholic hepatitis to grade severity in an Asian patient cohort.

findings of this model are as follows: (1) the MAGIC is the first prognostic model derived from an Asian population with AH; (2) it mainly focused on the prediction of natural outcomes of untreated patients with AH; (3) it firstly brought the prognostic role of hyperkalemia in AH to light, and most importantly; and (4) the spontaneous evolution in bilirubin levels is incorporated into this new model, emphasizing the importance of early amelioration of liver function in relation to the improvement of survival. However, this model should be further validated in other ethnic populations with severe ASH.

Corticosteroids seem to improve survival outcomes in patients with severe ASH without specific contraindications such as gastrointestinal bleeding, hepatorenal syndrome (HRS), uncontrolled infection, hepatitis B virus infection, and pancreatitis[14,15]. A recent meta-analysis of individual patient data from 5 randomized controlled trials demonstrated that a 28-d survival rate was higher in corticosteroid-treated patients than in placebo-treated ones (80% *vs* 66%)[26]. MDF, GAHS, and MELD at baseline assist in defining severe ASH and guiding when to initiate steroid treatment. On the other hand, early change in bilirubin level, *in vitro* resistance to steroid, and the Lille score at day 7 allow us to decide on the responsiveness to corticosteroids and whether to stop corticosteroids during steroid treatment[9,27-29].

Recently, an alcoholic hepatitis histologic score (AHHS) has been suggested to predict survival outcomes accurately in patients with biopsy-proven, ASH[30]. The AHHS is calculated by grading the extent of fibrosis, the degree of neutrophilic infiltration, bilirubinostasis patterns, and megamitochondria[30]. In particular, the pattern of bilirubinostasis was closely associated with the development of bacterial infections during hospitalization[30,31].

TREATMENT

General therapeutic measures

Alcohol abstinence is the linchpin of therapy for AH, since abstinence failure increases mortality rates among those with AH[32]. However, anti-craving drugs such as disulfiram, naltrexone, and acamprosate are not routinely recommended to patients with severe AH due to the risk of potential hepatotoxicity. Although an anti-craving medication is not promptly given to patients hospitalized for severe AH, an abstinence treatment should be considered to reduce the recurrence of alcohol use disorders after recovery of liver function. Baclofen could effectively suppress a craving for alcohol and keep an abstinence from alcohol in patients with alcoholic cirrhosis without incurring hepatotoxicity; however, additional research is needed to prove an anti-craving efficacy in those with severe AH[33].

Patients with AH often suffer from serious malnutrition resulting from promiscuous eating habits, alcohol-related diarrhea, decreased small bowel absorption capacity, anorexia, and an excessive catabolic state, which is directly related to increased mortality[34]. Accordingly, most of them require nutritional support including the adequate calorie and protein supply as well as vitamin B and mineral repletion along with dextrose water infusion. In addition, when oral feeding is not well tolerated in patients with AH, they often need fat-soluble vitamin supplementation and enteral nutrition. However, there was no significant difference of a 1-mo mortality rate in a previous study comparing enteral nutrition and corticosteroids in patients with severe AH[35]. Nonetheless, further studies are warranted to evaluate the impact of the combination treatment on survival, because early death was more frequent in the enteral nutrition group and late mortality was higher in the steroid-treated group.

In patients with severe AH, renal impairment is a frequently accompanied symptom during hospitalization and also represents an important predictor of infection and survival. The most common cause of acute renal dysfunction is HRS. To prevent HRS, nephrotoxic drugs such as nonsteroidal anti-inflammatory drugs, aminoglycoside, diuretics, and contrast dye should be avoided and volume expanders including albumin and fresh frozen plasma might be administered. Bacterial infection is frequent but difficult to diagnose, since SIRS criteria are often associated with sterile inflammation in ASH. Infection is commonly seen in around 25% of patients with severe ASH at admission and another quarter finally become infected while receiving corticosteroids during admission[36]. Thus, infection and renal failure in ASH have a major impact on survival and should be screened, prevented, or treated at all time-points. Empirical use of antibiotics, although widely instituted, is not routinely warranted. Recent data demonstrated that corticosteroids are not contraindicated for the treatment of ASH after a complete control of infection[36]. Infection developed

during steroid treatment, however, was not the result of immunosuppression by corticosteroids but that of non-response to corticosteroids suggesting severe liver impairment[36]. Such being the case, empirical antibiotic treatment may be more beneficial to steroid responders rather than non-responders by improving survival only in the former.

Specific therapies

Corticosteroids: Apart from general therapeutic measures, specific therapies are indicated for patients with severe AH (MDF $\geqslant$ 32) who are at high risk of early death according to clinical prognostic scores[26]. The impact of corticosteroid treatment on survival in those with severe AH has been under debate for the last three decades because of heterogeneity of the study design among different studies and selection bias from ambiguous diagnostic criteria lacking histologic confirmation. Moreover, the mechanisms underlying corticosteroid treatment for AH remain largely unknown. A recent study has carefully examined the effects of prednisolone on liver injury and regeneration in several experimental models regarding alcoholic liver injury[37]. In general, corticosteroids suppress inflammatory and immune-mediated hepatic destruction, but their marked, anti-anabolic effect may suppress regeneration and slow healing by inhibiting expression of genes (*i.e.*, *pSTAT3*) regulating the proliferation and repair of hepatocytes[37]. This study may give some new insights on prednisolone treatment for AH. In a recent meta-analysis, patients allocated to corticosteroid treatment (40 mg/d for 28 d) had a higher 28-d survival rate than those allocated to non-corticosteroid treatment[26]. Corticosteroids have now become a first-line therapy for biopsy-proven, severe ASH. Steroids may be sometimes deleterious in conditions other than ASH, which represent 10%-30% of patients with a clinical diagnosis of AH, dominated by infection-related decompensation. Moreover, the treatment of non-severe forms of AH by corticosteroids is not recommended. Thus, the effect of corticosteroids on survival seems to be restricted to biopsy-proven, severe ASH. Approximately, 60% of patients with advanced forms of ASH might benefit from corticosteroid treatment. Thus, early recognition of non-responders to corticosteroids (40% of the patients) is essential to define stopping rules and minimize the unnecessary exposure to corticosteroids[9]. A Lille score $\geqslant$ 0.56 at 7 d upon corticosteroids is defined as non-response to steroids. In non-responders, a 28-d survival rate was no more than 50% despite the continued treatment of corticosteroids[26].

Pentoxifylline: Pentoxifylline shows an antioxidant effect and weakly inhibits tumor necrosis factor-alpha (TNF-α) synthesis. In patients with severe ASH receiving pentoxifylline, a 6-mo survival rate was higher than in those treated with placebo[38]. The survival benefit was attributable to a lower incidence of HRS. However, this beneficial effect was challenged by two recent meta-analyses demonstrating that pentoxifylline decreased the risk of fatal HRS but did not improve survival significantly, although it remains inconclusive[39,40]. Recently, a Korean multicenter study group has made a head-to-head comparison between pentoxifylline and prednisolone[41]. The results demonstrated that the efficacy of pentoxifylline was not statistically equivalent to that of prednisolone in terms of 6-mo survival, supporting prednisolone as a preferred treatment option for severe AH. However, in patients with severe AH and contraindications to corticosteroids, pentoxifylline still can be considered as an alternative therapeutic option[14,15]. In a recent prospective trial including 270 patients with severe ASH, the combination of pentoxifylline and prednisolone did not bring any significant survival benefit over prednisolone alone[42]. However, a limitation of this study was that they failed to include a treatment arm receiving only pentoxifylline[43]. To overcome this limitation, a large randomized trial with a sufficient sample size is ongoing in the United Kingdom comparing pentoxifylline with corticosteroids or a combination of both in patients with severe AH[44]. Finally, in patients with severe ASH and non-response to corticosteroids based on the Lille model, an early switch to pentoxifylline did not improve the survival outcome[45]. Collectively, pentoxifylline has no additional beneficial effect in combination with corticosteroids in patients with severe ASH and also pentoxifylline alone is ineffectual in non-responders to steroids.

***N*-acetyl cysteine:** Recently, the combination treatment with *N*-acetyl cysteine (NAC), an antioxidant and prednisolone significantly reduced a 1-mo mortality rate compared with prednisolone alone by preventing HRS and infection, although the difference was no longer statistically significant at 3 and 6 mo[46]. However, given the trend toward improved survival in those treated with NAC, additional studies are required to determine the optimal dosing schedule and treatment duration of NAC.

Anti-TNF agents: Since strong evidence supported a central role for TNF-α in several experimental models of ALD, a randomized controlled study in patients with severe ASH tested infliximab in combination with corticosteroids[47]. In fact, the treatment aimed at blocking TNF-α, compared to placebo, was associated with a higher probability of severe infection and mortality[47,48]. Presumably, prolonged or excessive TNF blockade may cause profound immunosuppression and negatively impact liver regeneration[49-51].

Liver transplantation: AH is not considered as a usual indication for liver transplantation (LT). This is related both to the fact that most patients with AH will recover for at least 6 mo after abstinence, and to the "6-mo abstinence rule"[52]. The 6 months' abstinence rule, although socially acceptable and associated with low harmful alcohol relapse, can be replaced with other elements predictive of abstinence such as social and familial support and absence of psychiatric, addictive disorders[52]. The Lille model now allows the early identification of non-re-

Table 2 Summary of potential molecular targets and novel targeted therapies for alcoholic hepatitis

Key element of the pathogenesis	Treatment	Effect	Clinical trial
FXR dysregulation	OCA[67]	FXR agonist	Moderately severe AH (placebo *vs* OCA)
Altered gut integrity	Zinc[68]	Restoration of gut integrity	Severe AH
	LGG[69]	Probiotic effect	Mild to moderate AH (placebo *vs* LGG)
	Rifaximin[70]	Intestinal decontamination	Severe AH (steroid *vs* steroid + rifaximin)
Innate immune activation	Imm 12-E[71]	Anti-LPS antibody	Severe AH (steroid *vs* steroid + low/high dose Imm 12-E)
	Anakinra[57,58,72]	IL-1RA	Severe AH (steroid *vs* anakinra + pentoxifylline + zinc)
	Rilonacept[57,58]	IL-1 inhibitor	Severe AH with response to steroid at day 7 (steroid *vs* steroid + rilonacept)
	Mycophenolate mofetil	IMPDH inhibitor	Severe AH without response to steroid at day 7 (standard of care *vs* steroid + mycophenolate)
Sterile necrosis and apoptosis	Emricasan[54]	Pancaspase inhibitor	Severe AH with steroid contraindications (placebo *vs* emricasan)
Impaired regeneration	G-CSF[63,64]	HPC mobilization	Severe AH without response to steroid at day 7 (placebo *vs* G-CSF)
	IL-22[59,73,74]	Hepatoprotective effect	Only preclinical studies

FXR: Farnesoid X receptor; OCA: Obeticholic acid; AH: Alcoholic hepatitis; LGG: Lactobacillus GG; LPS: Lipopolysaccharide; IL-1RA: Interleukin-1 receptor antagonist; IL-1: Interleukin-1; IMPDH: Inosine-5′-monophosphate dehydrogenase; G-CSF: Granulocyte-colony stimulating factor; HPC: Hepatic progenitor cell; IL-22: Interleukin-22.

sponders to steroids, only 25% of whom being alive at 6 mo. Recently, an early LT concept was suggested to those with a first episode of severe ASH not responding to steroids[53]. Explicit improvement of survival was observed in patients who received early LT compared to historical controls without response to steroids[53]. Obviously, early LT in ASH may be relevant only in highly selected patients with a first episode of severe ASH, a favorable addiction profile, and not responding to medical therapy.

Novel therapies

Despite the current specific therapies against AH, the overall prognosis of severe AH remains dismal. Owing to the scarcity of available therapeutic resources, undoubtedly, there is an urgent need for novel and innovative therapies to combat against severe AH. Over the past several decades, we have made great progress in grasping the clinical course of AH but not been capable of successfully identifying therapeutic targets. The failure of most clinical trials in AH results from a poor knowledge of the key disease drivers. Secondly, systemic large-scale studies are required before we can engage into targeted, therapeutic trials. Finally, all animal models used to test targets represent mild ALD but not severe liver disease that characterizes AH.

Thus, to settle the aforementioned issues, we are increasingly encouraged to conduct multi-center collaborative trials that use common protocols, include biomarkers, and address the spectrum of AH. To that end, recently, National Institute on Alcohol Abuse and Alcoholism has decided to support four AH consortia, which will explore translational studies and clinical trials for AH[54]. Clinical studies will collect and bank genetic or other biologic samples and consents to allow translational studies of basic mechanisms, genetics, epigenetics, and systems biology of AH severity and of treatment response. In parallel with that, several interventional trials are ongoing through multi-institutional consortia to test proof-of-concept for new therapies (Table 2)[54].

Scientific integration for developing new biomarkers and novel therapies for AH mainly focuses on several key elements of the pathogenesis of AH. Firstly, inflammation cascade and innate immune activation are demarcating features of severe AH compared to mild to moderate ALD[55-58]. The syndrome of AH results from severe inflammation and cytokine dysregulation[59,60]. Secondly, gut integrity is significantly altered in AH allowing pathogen-associated molecular patterns to enter the liver and systemic circulation and induce innate immune activation[61,62]. Gut-derived endotoxins and other bacterial products that trigger inflammation are a consequence of increased permeability and altered gut barrier function[62]. Thirdly, cell survival and death pathways contribute to liver dysfunction and the release of damage-associated molecular patterns that further fuel inflammation including hepatocellular apoptosis, sterile necrosis, and injury[61]. Finally, hepatocellular regeneration is profoundly impaired in patients with severe ASH with liver failure. In this regard, it is therapeutically important to characterize the mechanisms of the poor hepatocyte regeneration and promote the differentiation of progenitor cells into functional mature hepatocytes[63-66].

CONCLUSION

There is a pressing need for better definitions to distinguish AH from other clinical syndromes. The definitions need to be related to risk and outcomes, to improve clarity of taxonomy, reduce problems with basic *vs* clinical classification, and aid in treatment decisions. To standardize the nomenclature of AH, we should compare the clinical, analytical, and molecular characteristics of early ASH that is completely asymptomatic with those of classical AH that appears in patients with jaundice and/or decompensation. Alcohol abstinence is the sine qua non of therapy for AH, and, in the milder forms, is prerequisite to clinical recovery. Severe ASH may progress to multi-organ failure and, in particular, renal impairment and infection are the most worrisome complications requiring screening, prevention, and treatment. Clinical

prognostic scores such as MDF and MELD are useful tools to determine whether to initiate steroids and the Lille model at day 7 can be applied to assess responsiveness to steroids as stopping rules. Pentoxifylline can be alternatively used as a first-line therapy in severe ASH patients with contraindications to steroids. However, pentoxifylline provides no additional beneficial effect to patients with severe ASH receiving corticosteroids. Early switch to pentoxifylline either does not significantly improve survival in non-responders to steroids. Convincingly, future studies should include homogenous population and direct to AH patients with intermediate severity and partial or non-responders to steroids. Strategically, we should explore novel therapeutic targets to restore altered gut mucosal integrity, suppress inflammation based on cytokine profiles, promote hepatic regeneration, and limit innate immune responses in severe ASH.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (6): Liver transplantation

Difficulties in diagnosing acute kidney injury post liver transplantation using serum creatinine based diagnostic criteria

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Abstract

Renal function in patients with advanced cirrhosis is an important prognostic factor for survival both prior to and following liver transplantation. The importance of renal function is reflected by the introduction of the model for end stage liver disease (MELD) score, which includes serum creatinine. The MELD score has been shown to predict the short term risk of death for transplant wait listed patients and is currently used by many countries to allocate liver transplants on the basis of severity of underlying illness. Changes in serum creatinine are also used to stage acute kidney injury. However prior to liver transplantation the serum creatinine typically over estimates underlying renal function, particularly when a colorimetric Jaffe based assay is used, and paradoxically then under estimates renal function post liver transplantation, particularly when immunophyllins are started early as part of transplant immunosuppression. As acute kidney injury is defined by changes in serum creatinine, this potentially leads to over estimation of the incidence and severity of acute kidney injury in the immediate post-operative period.

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Key words: Serum creatinine; Acute kidney injury; Liver transplantation

Core tip: Acute kidney injury is defined and severity graded based on changes in serum creatinine. Increasing concentrations of bilirubin interefere with laboratory determination of creatinine and reduce creatinine estimations. Post transplantation serum creatinine increases due to a combination of fall bilirubin and the loading doses of calcineurin inhibitor immunosupressants. This combination leads to an over estimation of the lesser grades of acute kidney injury post liver transplantation.

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WHY IS PERI-OPERATIVE RENAL DYSFUNCTION IMPORTANT IN LIVER TRANSPLANT RECIPIENTS?

Renal dysfunction is strongly associated with increased risk of mortality in patients with advanced chronic liver disease both awaiting liver transplantation (LT) and also peri-operatively[1-4]. Indeed renal function as determined by estimation of the serum creatinine concentration has been included in the model for end stage liver disease (MELD) score, which predicts the likelihood of death within 3 mo for patients wait listed for liver transplanta-

Table 1 Definitions of acute kidney injury using changes in serum creatinine between the Risk Injury Failure EndStage[7], Akute Kidney Injury Network[8] and Kidney Disease Improving Global Outcomes[9] criteria

Criteria	RIFLE[7]	AKIN[8]	KDIGO[9]
Date of release	2004	2007	2012
Time interval	Diagnosis and Staging: Within 1-7 d and sustained more than 24 h	Diagnosis: Within 48 h Staging: 1 wk	Diagnosis: 50% increase within 7 d or ⩾ 0.3 mg/dL (26.5 μmol/L) within 48 h
Stage 1 or R	Increased SCr 1.5-1.9 times baseline	Increased SCr 1.5-1.9 times baseline or ⩾ 0.3 mg/dL (⩾ 26.5 μmol/L) increase	Increased SCr 1.5-1.9 times baseline (7 d) or ⩾ 0.3 mg/dL (⩾ 26.5 μmol/L) increase (48 h)
Stage 2 or I	Increased SCr 2.0-2.9 times baseline	Increased SCr 2.0-2.9 times baseline	Increased SCr 2.0-2.9 times baseline
Stage 3 or F	Increased SCr 3.0 times baseline, or Increase in SCr ⩾ 4.0 mg/dL (350 μmol/L) with an acute rise of ⩾ 0.5 mg/dL (44 μmol/L)	Increased SCr 3.0 times baseline, or Increase in SCr ⩾ 4.0 mg/dL (350 μmol/L) with an acute rise of ⩾ 0.5 mg/dL (44 μmol/L)	Increased SCr 3.0 times baseline, or Increase in SCr ⩾ 4.0 mg/dL (350 μmol/L)

SCr: Serum creatinine; RIFLE: Risk Injury Failure EndStage; AKIN: Akute Kidney Injury Network; KDIGO: Kidney Disease Improving Global Outcomes.

tion and is used by several countries to preferentially allocate organs to those with more severe disease[2]. Serum creatinine is also part of the United Kingdom End Stage Liver Disease (UKELD) score which similarly predicts 12 mo waiting list mortality[5]. As such accurate assessment of renal function is important, particularly for patients with underlying chronic kdney disease, for example, patients with non-alcoholic steatohepatitis (NASH), due to coexisting diabetic, hypertensive micro or macrovascular renal disease[4,6]. Hence these patients then develop more renal dysfunction after LT, which is associated with increased mortality.

CURRENT DEFINITIONS OF ACUTE KIDNEY INJURY

In order to standardize the definition of acute renal failure, now termed acute kidney injury (AKI), the risk injury failure loss of function and end stage renal failure (RIFLE) guideline criteria were developed[7]. These were subsequently revised by both the acute kidney injury network (AKIN)[8] and more recently by the kidney disease improving global outcomes (KDIGO) group[9] (Table 1). Although all three classifications define stages of severity of AKI by both urine ouput and serum creatinine concentration, in practice most studies have retrospectively used changes in serum creatinine to determine both the incidence and severity of AKI in peri-operative LT transplant recipients. Although these AKI classification systems report increasing mortality with increasing AKI severity, in keeping with other patient groups[10], the question arises as to whether they accurately detect acute kidney injury. In theory the diagnosis of AKI based on these classifications should be relatively straight forward as to whether patients post LT have a 50%, 200%, 300% increase in serum creatinine or an absolute rise above a critical threshold to make the diagnosis of AKI and award an AKI classification (Table 1) Whereas the major hurdle in general medical or surgical practice is determining the "true" baseline serum creatinine measurement upon which to evaluate subsequent changes, all LT patients will have a pre-operative measurement, and initial daily post-operative serum creatinine estimations. So it would appear a simple matter of cataloguing changes in serum creatinine post-operatively to determine the incidence and severity of AKI post LT.

However serum creatinine estimations typically over estimate "true" renal function pre-operatively[11], and then under estimate renal function post-operatively so potentially increasing the reported incidence of AKI.

WHY DOES SERUM CREATININE OVER ESTIMATE RENAL FUNCTION PRE-OPERATIVELY?

Creatinine is non-enzymatically converted from creatine in muscle. Creatinine is predominantly synthesized in the liver. As such patients with chronic liver disease awaiting LT typically have reduced creatine synthesis due to the combination of reduced dietary protein intake and chronic liver disease. The conversion of creatine through to creatinine depends upon both muscle mass and muscle turnover. Patients wait listed for LT are at increased risk of sarcopenia (muscle wasting) and typically take less exercise than healthy controls, so have a lowered conversion of creatine to creatinine[12]. In addition as creatinine is measured as a concentration, then as many patients with chronic liver disease have oedema, with ascites this results in a larger volume of distribution of creatinine in the body and a lower serum creatinine concentration[13]. Serum creatinine may also be affected by the concomitant prescription of drugs, such as calcitriol which affect the renal tubular secretion of creatinine[14].

Serum creatinine estimations tend to overestimate glomerular filtration rate (GFR) in patients with chronic liver disease, as the most commonly used laboratory method is a colorimetric assay which is subject to interference by chromogens, including bilirubin (both conjugated and unconjugated). As such these chromogens lower the measurement of creatinine, so making serum creatinine an even less precise surrogate of GFR in jaundiced patients. There have been several attempts to improve the accuracy of the Jaffe assay in an attempt to reduce interference from chromogens, such as bilirubin, glucose, uric acid, ketoacids, pyruvate, and some antibiot-

Table 2 Cohort of 329 adult patients transplanted for advanced cirrhosis

	RIFLE[7]	AKIN[8]	KDIGO[9]
Stage 1 or R	53	93	97
Stage 2 or I	28	28	28
Stage 3 or R	8	8	8
Stage 3 initiation of RRT	17	17	17

Changes in renal as assessed bt RIFLE, AKIN and KDIGO criteria for acute kidney injury for changes in serum creatinine. RIFLE: Risk Injury Failure EndStage; AKIN: Akute Kidney Injury Network; KDIGO: Kidney Disease Improving Global Outcomes; RRT: Renal replacement therapy.

ics[15]. These include acid blanking and absorption techniques with Fuller's earth or Lloyd's reagent, and delayed rate reactions. Initially these were laborious and time consuming so unsuitable for routine use. However the newer generations of chemical pathology laboratory multichannel analyzers now often routinely incorporate modified delayed rate or blank correction creatinine assays. Another modification, the kinetic alkaline picrate method produces a differential rate of colour change between creatinine and non-creatinine chromogens. Enzymatic methods to determine serum creatinine, using creatininases and creatininase hydrolases have been shown to be more reliable and less affected by chromogens[16], but are generally much more expensive and as such has not been widely introduced into routine clinical practice. To put this into clinical perspective[17] the interference that occurs with serum bilirubin concentrations > 62 μmol/L (3.68 mg/dL), result in significant differences in reported serum creatinine values between different methods (modified Jaffe, compensated kinetic Jaffe, enzymatic and standard Jaffe), resulting in significantly different MELD scores. If differences in MELD score are only 1-2 points, then this would have little clinical consequence, but differences of 3 or 4 points which are seen with bilirubin concentrations between 100 μmol/L (5.85 mg/dL) and 200 μmol/L (11.6 mg/dL) are clinically relevant. At even higher serum bilirubin concentrations (> 23.4 mg/dL), *i.e.*, those with the highest priority for LT. then this interference can result in differences in up to 7 MELD points As such the method used to estimate serum creatinine used in MELD scoring should be taken into account, as some patients inadvertently will be discriminated against with respect to others, in terms of priority for LT , when allocation is based on MELD score.

A further problem associated with accuracy and precision of serum creatinine measurements is a lack of universal standard for creatinine. For example in the United Kingdom there were 34 variations of the standard Jaffe reaction used by United Kingdom National Health Service (NHS) clinical chemistry laboratories. To standardize assays, all NHS laboratories were sent isotope dilution mass spectroscopy (IDMS) standards to develop their own correction factors for their creatinine assays. However IDMS standards do not allow for interfering chromogens and as such marked differences remain in serum creatinine estimations between UK NHS laboratories serving liver transplant centres[17,18].

Perhaps not surprisingly because of these multiple limitations of serum creatinine in estimating renal function in patients with advanced chronic liver disease a meta-analysis proposed that GFR estimation by inulin clearance was the only way for accurate assessment of renal function[19], but unfortunately inulin clearance remains impractical for routine clinical use.

WHY DOES SERUM CREATININE UNDER ESTIMATE RENAL FUNCTION POST-OPERATIVELY?

Although creatinine excretion is predominantly by glomerular filtration, there is an additional amount of creatinine secreted by the renal tubule. As such drugs which cause a reversible reduction in glomerular filtration can lead to an increase in serum creatinine, without necessarily causing renal damage. In the post-operative LT patient these would include non-steroidal anti-inflammatories given for post-operative analgesia, and on-going prescription of pre-operative antihypertensive medications, not just angiotensin converting enzyme inhibitors, angiotensin receptor blockers and renin inhibitors. However the drugs most likely to reduce GFR in the immediate post-operative period are the immunophyllins, particularly tacrolimus. In addition to reducing GFR, immunophyllins also reduce renal tubular creatinine secretion by inhibiting cyclooxygenase 2 in the renal medulla so causing renal tubular ischaemia[20].

Hence serum creatinine tends to overestimate renal function prior to LT, and then under estimate renal function post operatively. Thus when using the current definitions of acute kidney injury based on changes in serum creatinine there is a tendency to overestimate both the incidence and severity of acute kidney injury post LT (Figure 1, Table 2). This is most marked for lesser degrees of acute kidney injury, and most noticeable between the RIFLE and other scoring systems (Table 2), due to the differences in definitions with RIFLE requiring a 50% increase compared to AKIN and KDIGO which only require an absolute increase of 0.3 mg/dL. So that switching from a high to a low serum bilirubin post transplantation and starting immunophyllin immunosuppression may be sufficient to cause a minor increase in measured serum creatinine to be classified as acute kidney injury stage 1 by AKIN and KDIGO, but less than the 50% increase required by RIFLE.

Altering peri-operative immunosuppression protocols to delay or avoid the initial use of immunophyllins may help to reduce kidney injury, by using monoclonal antibodies, such as simulect and CAMPath-1, particularly in those with NASH and other patients with pre-existing chronic kidney disease. Lower targets for tacrolimus trough doses have also recently been shown to improve graft survival and reduce both acute and chronic renal impairment[21,22]. Thus, risk modification is needed to optimize renal function in the pre, peri and postoperative

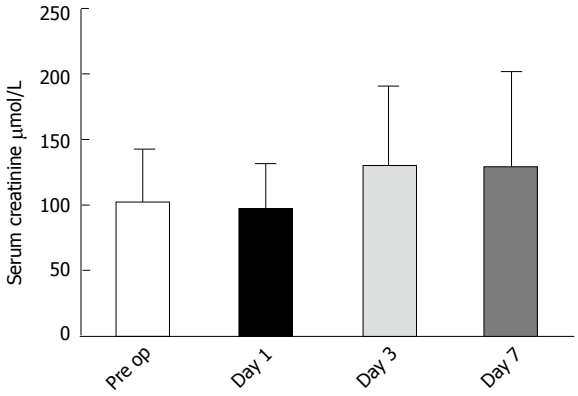

Figure 1 Cohort of 329 adult patients undergoing liver transplantation at the Royal Free Hospital. Serum creatinine measured using an enzymatic method shows a significant increase between the pre-operative and 1st post-operative day and the 3rd and 7th post-operative days respectively. Only 2.4% developed acute kidney injury stage 3 on serum creatinine criteria alone, suggesting that the most probable cause for the significant increase was due to changes in serum creatinine measurement due to a reduction in bilirubin and other chromagens, and the use of immunophyllins as immunosuppressive agents.

management of LT candidates: which may prevent or delay post-LT end stage renal disease[22].

WHAT ARE THE ALTERNATIVES TO MEASURING SERUM CREATININE?

Exogenous markers which are only cleared by the kidney are the most accurate methods for determining renal function. Inulin clearance remains the gold standard for measurement of renal function but cost and technical difficulties limit its use for routine practice[19]. Other direct methods of measuring GFR include exogenous radiolabelled substances (^{51}Cr-ethylene diamine tetra acetic acid (EDTA), ^{99m}Tc-diethylenetriamine pentaacetate (DPTA) and ^{51}I-iothlamate) or non-radioactive agents (iohexol or iothalamate)[23]. However these methods have typically not been extensively validated in patients with cirrhosis and ascites. As such the British Nuclear Medicine Society Guidelines stated that liver failure, ascites, oedema and low clearance status may produce inaccurate clearance values[24]. Following injection there will be an initial redistribution from plasma into the ascites, and then a later re-equilibration from the ascites back into the plasma. As such ascites has been reported with increased clearances of 16-20 mL/min based on compartmental models[24]. To overcome these difficulties delayed sampling has been used to improve the calculation of the decay slope and time zero[25]. These isotope and radiocontrast techniques correct measurements of glomerular filtration for body surface area, which is calculated using equations based on height and weight. The presence of ascites and changes in body composition[26-28], with loss of muscle and fat mass change the normal relationship between calculated body surface area and muscle mass, and so add errors to the determination of GFR. Ideally these methods can be used for pre-operative assement of renal function, which can then be used to stratify patients for risk of acute kidney injury post LT and individualise immunosuppression policies. However there use in the immediate post LT period is unclear when renal function is changing.

ESTIMATION OF CREATININE CLEARANCE BY URINE COLLECTIONS

Creatinine clearance, using 24 h urine or shorter timed collections was the traditional method for assessing renal function. However, creatinine clearance underestimates GFR in children and when the serum creatinine levels are high the relative proportion of creatinine secreted by the renal tubules is greater[29] (Table 3). In healthy adults, creatinine clearance typically overestimates "true" GFR based on inulin clearance. Limitations are associated not only with the use of serum creatinine, measurements but also tubular creatinine secretion, which increases with underlying chronic kidney disease, proteinuria, drugs and also extra-renal elimination of creatinine by micro-organisms in the gastro-intestinal tract[14]. Pre-operatively , there may be up to 25% variation in GFR estimation based on creatinine clearance[29], due to incomplete urine collections, timing errors, errors in urine volume measurement, variations in tubular excretion or re-absorption of creatinine, serum creatinine dilution due to increased fluid retention and other unpredictable factors. Due to these multiple errors, there is no evidence that creatinine clearance is superior to serum creatinine in determining renal function in cirrhosis.

MATHEMATICAL ESTIMATIONS OF GLOMERULAR FILTRATION RATE

To overcome some of the limitations of 24 h urine collection, a number of different mathematical formulae have been developed, which incorporate serum creatinine to provide an estimate of GFR (eGFR). However these formulae were developed from a stable chronic kidney disease population, and not for patients with chronic liver disease, or for patients with changing renal function in the post-operative LT period. Although these formulae are increasingly being used in the intensive care setting they have not been validated. Currently used formulae include the Cockcroft-Gault (C-G)[30] and Modification of Diet in Renal Disease (MDRD)[31] formulae. The C-G formula requires serum creatinine, weight, gender and age whereas the MDRD formula incorporates serum creatinine, ethnicity, gender and age (MDRD-4), or creatinine, ethnicity, gender, age, albumin and urea (MDRD-6). Thus, in contrast to C-G formula, a body weight variable (which is difficult to assess as lean body mass in ascitic and malnourished patients) is not needed, and the MDRD equations use ethnicity, gender and age and then adjusts for 1.73 m^2 body surface area (without any assessment of height or weight). In cirrhosis, although there is discrepancy when compared to ^{125}I-iothalamate[32], the MDRD-6 equation is considered a more accurate for-

Table 3 Comparison of the established methods for assessing renal function in clinical practice

	Advantages	Disadvantages
Serum marker		
Creatinine	Widely available	Influenced by several factors unrelated to renal function, including dehydration and volume expansion, dietary protein, muscle mass, physical activity and thyroid hormones renal tubular secretion affected by chronic kidney disease, proteinuria and drugs not an early biomarker of acute kidney injury
Clearance of exogenous marker	"Gold standard"	Absence of standardization of the laboratory methods for jaundiced patients technical difficulties and expense make impractical for routine clinical practice stable renal function Less reliable in patients with oedema, ascites, pleural effusions and sarcopenia
Creatinine Clearance f (24 h urine collection)	? more accurate compared to Cr	Inconvenient for outpatientsoverestimates GFR in proteinuria chronic kidney disease influenced by muscle metabolism and diet, inflammatory disease and malnutrition Unexplained variation due to incomplete urine collection and errors in urine volume measurement overestimation of GFR in patients with cirrhosis
Mathematical formulae based on Cr	Easier method compared to 24 h urine collection	Not validated for patients with changing renal function (acute kidney injury, muscle wasting disorders) Does not overcome the limitations in serum creatinine
C-G formula	Requires only gender, age, body weight	Difficult to determine body weight in patients with ascites and post LT
MDRD formula	Body weight is not needed ethnicity, gender and age are taken into account	Has not been validated in patients with chronic liver disease 6-variables formula: needs albumin, urea Only validated in stable chronic kidney disease patients

GFR: Glomerular filtration rate; Cr: Serum creatinine; C-G: Cockcroft-Gault; MDRD: Modification of Diet in Renal Disease; CKD: Chronic kidney disease; LT: Liver transplantation.

mula, compared to C-G, possibly because it incorporates urea and albumin, which are abnormal in cirrhotics and it excludes body weight, a variable which may be difficult to determine in malnourished patients with ascites[28]. However the MDRD-4 formula is the formula reported by most laboratories, as it was equally accurate as the original six-variable formula in screening for patients with chronic stable kidney disease. In cirrhosis, C-G and both MDRD formulae typically overestimate true GFR, particularly in those patients below 50 years old or those with ascites[33]. Due to inaccuracies of the MDRD-4 equation in determining renal function in patients with an eGFR > 60 mL/min, a new creatinine-based equation known as the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, using the same variables with MDRD-4 formula, has been proposed, but its superiority in patients with cirrhosis has not been validated[34]. Nevertheless, the use of such formulae does not overcome the limitations in serum creatinine measurement. It has been recommended that creatinine results used for calculating eGFR should be traceable to an IDMS reference method[34], but the IDMS standards do not correct for the effects of chromogens. To overcome the problem that these formulae were derived from cohorts without liver disease new formulae for patients with cirrhosis have been proposed including adding the Child Turcot Pugh (CTP) score and ascites into the formula[35]. These newer formulae have been reported to show better agreement with "true" GFR, compared to the MDRD formulae, but require further external validation before they can be introduced into clinical practice.

ALTERNATIVES TO CREATINNE

Serum cystatin C

Serum cystatin C is an extracellular inhibitor of cysteine proteases[36]. It was originally thought that cystatin C was uniformly produced and secreted by all nucleated cells, but actually has a greater diurnal variation than serum creatinine. Cystatin C is freely filtered by the renal glomeruli and then taken up and catabolized in the proximal tubules. It was initially considered a more sensitive indicator of renal function compared to creatinine[37], in several disease groups including cirrhosis[38,39]. Consequently several Cystatin C based GFR equations, were derived[40,41]. More recently cystatin C has been recognized to be affected by numerous factors including inflammation[42] body composition, proteinuria, cardiovascular risk factors[43,44] infection, thyroid dysfunction, underlying malignancy, smoking and a number of drugs; including corticosteroids, cotrimoxazole, angiotensin converting enzyme inhibitors, and calcineurin inhibitors. Cystatin C has been reported to increase with severity of chronic liver disease[45] as it correlates with bilirubin, INR and CTP stage, and negatively with serum albumin and peripheral platelet count[46]. As cirrhosis evolves the increasing cystatin C values may be related to increased production, secondary to inflammation, or decreased clearance due to reduced renal function. The original cystatin C equations were all derived from non-liver disease populations[47,48]. Recentstudies have evaluated cystatin C GFR formulas in patients with cirrhosis[35]. One reported that although cystatin C formulas were more accurate than the creatinine formulas[49]. GFR estimations were significantly different

to inulin clearance. In the second study serum cystatin C formulas not only significantly overestimated renal clearance compared with ^{51}Cr-EDTA but did not provide any advantage over serum creatinine formulas[35], and serum cystatin C values were significantly affected by the presence of ascites. Although a third study reported cystatin C to more accurately represent renal function than serum creatinine[50]. There has recently been standardisation of serum cystatin C assays and more studies are required to try and develop specific GFR formulae for cystatin C in patients with cirrhosis. However as cystatin C is increased by inflammatory states, changes in cystatin C performs no better than serum creatinine in determing acute kidney injury in the immediate post operative lT period.

NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN

Acute kidney injury is a potentially life threatening complication in patients with cirrhosis. Neutrophil Gelatinase associated Lipocalin (NGAL) has been recently introduced as an early marker of tubular dysfunction in acute kidney injury. Several studies have reported that NGAL increases in urine and plasma shortly after injury to renal tubular cells and it can be used to aid the differential diagnosis between acute tubular necrosis and volume responsive causes of acute kidney injury in patients with chronic liver disease. Urinary and serum NGAL not only reflect renal tubular injury but are also markers of the host systemic inflammatory response, as NGAL is part of the innate immune response designed to restrict iron availability to invading micro-organisms. After initial promising reports of the superiority of NGAL to other acute kidney injury biomarkers including creatinine[51] more recent reports have failed to substantiate the earlier studies, especially when studies include patients with pre-existing chronic kidney disease.

NGAL has been evaluated in patients with cirrhosis[52]. Patients with kidney dysfunction irrespective of aetiology had greater serum NGAL levels compared to those without kidney dysfunction irrespective of the presence of ascites. Urinary NGAL levels were also increased significantly in patients with cirrhosis and acute tubular necrosis (median values 417, range 239-2242 μg/g creatinine) compared to those with other causes of acute impairment of kidney function, for example hepatorenal syndrome (not associated with active infections), pre-renal azotemia secondary to volume depletion, and chronic kidney disease. However, plasma levels of NGAL were not helpful in the differential diagnosis of kidney dysfunction, in particular reversibility of acute kidney injury. Urinary NGAL levels were found to be significantly increased with urinary tract infections, whereas plasma NGAL was not different in patients with and without bacterial sepsis. As such, NGAL did not aid the differential diagnosis between acute tubular necrosis and hepatorenal syndrome precipitated by infection, as NGAL levels increased in both groups. Thus, larger multicentre trials are awaited to determine whether urinary NGAL, and newer markers of acute kidney injury, such as kidney injury molecule 1 (KIM-1) and urinary IL-18 excretion have a role in diagnosing acute kidney injury in patients with chronic liver disease. Similarly studies in patients following LT have reported that NGAL rises in patients who develop acute kidney injury[53]. Although n serum NGAL may rise earlier than creatinine post LT, this may simply reflect the severity of the ischaemia-reperfusion injury and the initial dilutional effect of intra-operative fluid administration on serum creatinine. Additional studies are warranted to determine whether there is a clinical role for these newer biomarkers in the diagnosis of acute kidney injury following LT.

CONCLUSION

Renal dysfunction increases the risk for mortality in patients with chronic liver disease both prior to and post liver transplantation. Changes in serum creatinine are now used to define acute kidney injury. As such, although small changes in serum creatinine are linked to adverse outcomes, changes in serum creatinine concentration can be influenced by changes in hydration status[54], and in particular for the patient with cirrhosis a falling serum bilirubin post liver transplant can lead to an apparent increase in serum creatinine, simply due to loss of interference with the colorimetric assay, and secondly due to changes in intra-renal perfusion associated with immunphyllins, without necessarily implying acute kidney injury. As serum creatinine is likely to remain the routine clinical marker of kidney function, additional biomarkers are required to help differentiate between assay interference and reversible changes in renal function on one hand and acute kidney injury on the other.

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REVIEW

Laser ablation for small hepatocellular carcinoma: State of the art and future perspectives

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Abstract

During the last two decades, various local thermal ablative techniques for the treatment of unresectable hepatocellular carcinoma (HCC) have been developed. According to internationally endorsed guidelines, percutaneous thermal ablation is the mainstay of treatment in patients with small HCC who are not candidates for surgical resection or transplantation. Laser ablation (LA) represents one of currently available loco-ablative techniques. In this article, the general principles, technique, image guidance, and patient selection are reported. Primary effectiveness, long-term outcome, and complications are also discussed. A review of published data suggests that LA is equivalent to the more popular and widespread radiofrequency ablation in both local tumor control and long-term outcome in the percutaneous treatment of early HCC. In addition, the LA technique using multiple thin laser fibres allows improved ablative effectiveness in HCCs greater than 3 cm. Reference centres should be equipped with all the available techniques so as to be able to use the best and the most suitable procedure for each type of lesion for each patient.

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Key words: Liver; Hepatocellular carcinoma; Minimally invasive procedures; Laser; Laser ablation

Core tip: The aim of this review is to describe the basic principles, results in terms of safety and efficacy, and recent advancements in laser ablation (LA). This mini-invasive technique is a less known and few employed procedure as compared to radiofrequency ablation (RFA). However, according to published studies LA is as safe and effective as RFA. In the review the technique and potential advantages of LA are described. Our ambition is to provide the hepatologists, and other physicians, with an updated approach to this ablative technique.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a global health problem, ranking as the sixth most common malignancy and the third most frequent cause of cancer-related death worldwide[1-3]. Its incidence is rising, mostly due to the diffusion of hepatitis B or C virus infection, alcohol-related cirrhosis, and nonalcoholic steatohepatitis[2,4]. Its incidence increases with advancing age and is more common in males[5,6]. Thanks to semiannual surveillance of the high-risk population by ultrasound and alpha-feto protein, HCC is increasingly detected at early stage, when curative treatments can be employed[3,4,7,8]. Resection is the mainstay of treatment for patients with HCC solitary tumours, preserved liver function, or mild portal hypertension not suitable for liver transplantation (LT)[9-11],

the latter being the only cure of both HCC and underlying cirrhosis[12]. However, resection may be associated with significant morbidity as well as tumour recurrence, which occurs in about 70% of patients at 5 years[13-17]. When surgery is unfeasible, percutaneous or laparoscopic tumour ablation is the most widely used treatment that can achieve the complete local control of the disease in properly selected candidates[4,18,19]. This procedure is also cost-effective as compared to surgical treatments because it destroys only a minimal amount of liver parenchyma whilst reducing the number of hospitalizations[18,20,21]. Among the available local ablative techniques, laser ablation (LA) is a less investigated and little-used treatment. Our ambition is to provide hepatologists and other physicians with an updated approach to this ablative technique.

GENERAL PRINCIPLES

Laser source

In 1983, Bown[22] described for the first time the use of laser light to ablate liver tumours. Laser devices transform electrical energy into light energy, which interacts with tissue to produce heat and cause cell death[23]. Laser light can be delivered precisely and predictably into any location of the liver. Laser is an acronym for "light amplification by stimulated emission of radiation", a principle based on the spontaneous emission of characteristic photons by excited atoms. Because laser light is coherent and monochromatic, it can be highly collimated and focused and large amounts of energy can be transmitted over long distances without significant losses. The light produced is of a specific wavelength and defines the properties of the laser system and the extent of tissue penetration. Due to the optimal penetration of light in the near-infrared spectrum, neodymium-doped yttrium aluminium garnet (Nd:YAG) lasers with a wavelength of 1064 nm and diode with a wavelength of 800-980 nm are preferred for percutaneous LA[24]. The optical (scattering, reflections, and absorption), thermal (conductivity and thermal storage), and blood flow characteristic of the tissue govern thermal diffusion processes and define the temperature map within the laser-exposed area[25]. The extent and completeness of tumour necrosis depends on a balance between the power applied and tissue charring[26].

Laser transmission

Laser light is transmitted from the source to the patient through flexible optic fibres that have specially designed diffuser tips. An important role is played by the shape, size, and design of the fibre[27-29]. The most common types of fibre currently used are the bare-tip[30,31] and cylindrical diffusing quartz fibres[29]. For the ablation of large masses or multiple tumours located at different sites, beam-splitting devices allowing the simultaneous delivery of light into multiple fibres can be used[24,32,33]. Multi-fibre systems have a synergistic effect by reduced heat dissipation between fibres[24,32]. The use of water-cooled laser application sheaths allows operating at higher powers and makes large lesions faster. Lesion diameter approaches 5-8 cm[33-35] with minimal charring and carbonization[26,29,36-41]. Because there is no destruction of the fibre, multiple applications are generally quite easy and longer lesions can be generated by simply pulling the fibre back in the applicator or advancing it forward.

Role of imaging guidance

The ablation procedure is performed under conscious sedation and local anaesthesia. Real-time ultrasound (US), computed tomography (CT), or magnetic resonance imaging (MRI) are employed to guide either one or multiple thin needle-fibres[42,43] or a coaxial guide needle through tissue and into lesion[44,45]. Most patients are treated as day-cases in outpatient clinics. The number of treatment sessions varies according to the size and number of lesions. Follow-up evaluations are performed within 24 to 48 h or within 4 wk from procedure, and then at 3 to 6 mo intervals as is usual with other thermal techniques[42,44].

US is used for targeting and monitoring during the procedure, while CT is mainly used for post-treatment assessment. Heated tissue becomes hyperechoic because of water loss; this is most pronounced when there is tissue charring[46,47], particularly evident when using uncooled devices as in the laser technique with thin needle-fibres[31,42]. As it is well known, the main disadvantage of US guidance is that it is not suitable to accurately evaluate the temperature or the size of the ablative zone being created[47,48]. Otherwise, the contrast-enhanced US is useful to detect residual disease during procedure[49,50].

Real-time CT is unreliable for the detection of the early signs of laser-induced tissue injury. However, contrast enhanced CT 24 h after the procedure identifies coagulation zone as a not perfused area and correlates precisely with histology. The main role is the detection of residual or recurrent tumour following LA. Within a few days from treatment, the edges of ablation zone become indistinct due to inflammatory changes. During follow-up, local recurrences are easily visualized as contrast-enhancing foci adjacent to the necrotic area[30,51-53].

In contrast, MRI performed during LA allows the monitorization of the actual size and temperature of the ablation zone. MRI is the most accurate method for planning, monitoring, controlling, and assessing laser-induced coagulative necrosis[54-56]. LA power settings and session-treatment durations can be adjusted to obtain appropriate temperature elevations beyond tumour margins, thereby achieving a sufficient safety margin of necrosis. MRI is well suited to detecting residual undamaged tissue or local recurrence in the transition area[57]. This procedure is mainly used in combination with the high power water-cooled laser systems so that the treatment can be performed safely and with a better control of the extent of the ablated area[40,58]. MRI images can be acquired in near real time in any arbitrary plane. This has advantages in optimal planning of the procedure and in more accurately targeting the treatment volume and avoiding damages to critical structures[59]. After treatment delivery, changes

in parameters such as tissue perfusion or diffusion may be used in addition to routine relaxation mechanisms (T1 and T2 weighting) to visualize the extent of ablation. Thus, because modern LA delivery aims to generate lesions rapidly in tissue that has many connective heat sinks and critical structures (such as brain and prostate), the ability to visualize and often quantify tissue temperature changes can be crucial feedback to the safety, efficacy, and overall outcomes of the thermal procedures[24,59,60].

Indications

According to the procedure used and the accessible facilities, selection criteria vary among centers[31,42,44,53,61-65] being established on size, number, and site of HCC in patients who are considered not good candidates for resection or liver transplantation. Although lesions of up to 6 cm have been treated[43], patients eligible for LA are those whose tumours are in accordance with the Milan criteria, irrespective of their location[66-68]. In fact, cancers located near major vessels, bile ducts, bowel, or diaphragm can be ablated with caution with RFA[69] but they can be more safely treated both with MRI-guided technique[44] and, more easily, with very thin devices with a calibre of 0.7 mm (21 gauge)[42,43,53,65-68]. MRI-guided technique allows confident ablation of high-risk located lesions using the real time thermometry and multiplanar MRI targeting[44,58,61,63].

Effectiveness and outcome data

Several retrospective cohort studies have shown that LA is a safe and feasible procedure for the treatment of HCC[31,42-44,53,61-68]. Using multiple bare fibres introduced through 21-gauge needles positioned under US-guidance, the reported complete response rate ranges from 82% to 97%[66-68]. In lesions in high-risk sites, complete response is 95.5%[65]. In patients with monofocal HCC $\leqslant$ 4 cm or three nodules $\leqslant$ 3 cm each, reported cumulative survival rates at 3 and at 5 years range from 52% to 68% and from 15% to 34%, respectively[53,66-68]. Tumor size, tumor location, and complete ablation were the main factors affecting the outcomes. In a multicenter study, Child's class A patients had a 5-year cumulative survival of 41%; the median survival time was 65 and 68 mo in patients with tumor size $\leqslant$ 3 cm and $\leqslant$ 2 cm, respectively. The authors stated that the ideal candidates for LA are younger patients with serum albumin within the normal range and a tumor size $\leqslant$ 2 cm in whom it is very likely that complete ablation will be achieved. The median time to recurrence was 24 mo and the median disease-free survival time was 26 mo[68]. Like RFA and microwaves ablation (MWA), LA resulted safe and effective also in the treatment of cirrhotic patients awaiting liver transplantation[70].

Promising results have been reported with the use of water-cooled higher power MRI-guided LA. A very low local recurrence rate and a complete response rate reaching up to 98% in nodules $\leqslant$ 5 cm has been achieved with this technique[44,61]. In a study on 39 patients with 61 HCCs a complete ablation rate of 98% and a mean survival rate of 4.4 years were observed[61]. More recently, the same authors confirmed the high percentage of complete response in a cohort of 113 patients with 175 HCCs $\leqslant$ 5 cm followed for a period of over 15 years; 75% of the lesions were located at high-risk sites and median survival was 3.5 years[44].

To date there is only one controlled study comparing LA with RFA in treating a small cohort of patients (81 cirrhotic patients with 95 biopsy-proven HCCs) with early stage HCC (nodule $\leqslant$ 4.0 cm or three nodules $\leqslant$ 3.0 cm each). Thin multiple fibre technique to perform LA and single or cluster 17-gauge cool-tip electrodes for RFA were employed. The authors found LA and RFA to be equally effective; but fewer treatment sessions were needed in RFA group to achieve complete response. Neither significant differences in survival rates between the two methods nor significant complications were observed in both groups[71].

In a randomized prospective trial in a single centre with three years of follow-up being evaluated for final publication, the authors treated 140 patients with 157 biopsy-proven HCCs to compare LA and RFA (70 patients with 77 nodules and 70 patients with 80 nodules, respectively). Median follow up in RFA and LA groups was 21 and 22.5 mo, respectively. Complete response was observed in 97.2% and in 95.8% of RFA and LA group patients, respectively. Median time to tumour recurrence was 25.6 and 37.8 mo in RFA and in LA groups, respectively (P = 0.129). Estimated probability of survival at 1, 2, and 3 years was 94%, 88%, and 66% in RFA group and 94%, 81%, and 59% in LA group, respectively (P = 0.693). No major complications or significant treatment-related morbidity were observed in both groups. The authors concluded that LA was non inferior to RFA either in obtaining the complete ablation of HCC nodules or in long-term outcome[72].

Use in combination with other treatment

Multi-ablation therapy consisting of LA before trans-arterial-chemo-embolization (TACE) has been effective in large HCCs with a mean diameter of 5.2 cm (range, 3.1-9.6 cm)[73], with complete response achieved in 90% of the large tumors. Fifteen additional synchronous small HCC $\leqslant$ 3 cm in 11 patients were completely ablated (100%) with LA alone. The survival rate was overall 40% at 3 years and 60% in Child class A patients. The 1-, 2-, and 3-year local recurrence rate for the main tumors was 7% annually while the 1- and 2-year cancer-free survival rates were 74% and 34%, respectively. The rationale of this study was that LA reduces tumor volume within the range of TACE effectiveness and at same time can achieve complete destruction of large lesions with a lower number of TACE sessions (in 70% of patients only a single TACE session was done). Recently, the introduction in clinical practice of a novel needle guide system makes it possible to achieve complete ablation of nodules

Table 1 Studies reporting the outcome of Laser Ablation for small hepatocellular carcinoma

Ref.	Pts/Tumors no	Tumor size (cm) mean	Complete ablation[a], %	Local recurrence rate, %	Overall survival %		3-yr disease-free survival %	Major complication rate[b], %	Mortality rate, %	*P* value
					3-yr	5-yr				
Giorgio *et al*[31] (2000)	77/85	≤ 4.0[c]	82[f]	1.1				3.9[d]	1.3[d]	
Pacella *et al*[73] (2001)	30/30	> 5.0	90[f] (+ TACE)	7	40			0	0	
	30/15	≤ 3.0	100[f]	0				0	0	
	Child-Pugh A				60					0.001
	Child-Pugh B				0					
Pacella *et al*[66] (2001)	74/92	≤ 4.0[c]	97[f]	6	68	15		0	0	
	Child-Pugh A				73	31				0.052
	Child-Pugh B[7,8,9]				68	0				
Eichler *et al*[61] (2001)	39/61	≤ 5.0[e]	97.5[g]	0	4.4 yr			0	0	
Pacella *et al*[42] (2005)	82/99	≤ 4.0[c]	90.9[f]	8.8				1.5	0	
Francica *et al*[64] (2007)	148/169	≤ 4.0[c]	82[f]	14.7	52	27		0.6	0.6	
		≤ 2.0	95[f]	5						
		≤ 3.0	89[f]	15						
		> 3.0	74[f]	26						0.001
	Well-differentiated	≤ 3.0		0	58					
	Poorly-differentiated			25						0.008
Pacella *et al*[68] (2009)[1]	432/548	≤ 4.0[c]	79.6[f]	20[2]	61	34		1.6	0.2	
		≤ 2.0	85.1[f]		41					
	Child-Pugh A				63					
	Child-Pugh A	≤ 2.0			68[3]					
Francica *et al*[53] (2012)	106/116[4]	≤ 4.0[c]	92.2[f]	10.6				0.9	0.5	NS
	58/66	≤ 4.0[c]	95.5[f]							
Francica *et al*[65] (2012)	116/132	≤ 4.0[c]	100[f]	18.0	57	29				0.029[5]
				2.2[5]						
Eichler *et al*[44] (2012)	113/175	≤ 5.0[e]	98[g]	1.1	54	30		0	0	
Di Costanzo *et al*[43] (2013)	104/116	≤ 6.0	87.6[f]	16						
		≤ 5.0	91.7[f]							
Di Costanzo *et al*[72] (2013)[6]	70/80 (LA)	≤ 5.0[6]	96.3[f]	23.9	66		42	0	0	
	70/77	≤ 5.0[6]	97.4[f]	25.7	59		43	0	0	0.693

[a]Calculated per tumor; [b]Calculated per patient; [c]Single tumor ≤ 4 cm or ≤ 3 nodules each ≤ 3 cm; [d]In patients with Child-Pugh C; [e]Single tumor ≤ 5 cm or multiple ≤ 5; [f]With bare fibers; [g]With water-cooled fibers; [1]Multicentric retrospective study; [2]Local and distant; [3]In pts Child-Pugh class A well-differentiated tumor; [4]At risk site; [5]Only if the ablative margin was ≥ 7.5 mm; [6]RCT with Milan criteria; [7,8,9]Refers to the Child-Pugh class. NA: Not available; NS: Nonsignificant; CR: Complete response.

up to 5 cm in a single session in 91.7% of cases without resorting to combined treatment[43]. All data reported above are summarized in Table 1.

LA may also be combined with other modalities to achieve an increased volume of tumor necrosis. Zou *et al*[74] demonstrated that combined therapy with PEI immediately followed by LA resulted in a significantly larger volume of coagulation zone with reduced residual tumor volume on rabbit VX2 liver tumors. These authors hypothesized that tissue destruction by ethanol may have resulted in increased thermal conduction. In addition, the sclerosis and/or destruction induced on small vessels by PEI causes a reduction of the heat-sink effect and thus an enhancement of laser ablation effect. To date there are no clinical trials with this technique.

Complications

Arienti *et al*[75] performed a multicenter study involving nine centers in Italy with 520 patients who underwent 1064 nm laser sessions for 647 HCCs. Analyzing 90 factors for each record, including tumour characteristics, the authors reported a major complications rate of 1.5% (0.8% death rates) and a minor complications rate of 6.2%. These authors enrolled in their retrospective and prospective study patients with HCC nodules of any size [387 (60%) small, 180 (28%) intermediate, and 74 (11%) large] including 29 (5.9%) patients in Child's class C and 72.1% of patients with portal hypertension. Major complications were associated with excess energy deposition and high-risk nodule locations. Minor complications proved to be associated with excess energy, high bilirubin level, and low prothrombin time. The authors who use MRI-guidance and high-calibre water-cooled devices reported no major complications or cases of mortality in 152 patients treated using large bore water-cooled devices[44,61]. In these series, no case of tumour seeding was observed.

Costs

Using multiple small-bore needles, the price of each laser disposable kit including a needle and a fiber is about €300 (US$ 400). Therefore, the cost of a single LA session varies in relation to the number of devices used: one kit is required for nodules ≤ 1.0 cm; 2 kits for nodules ranging from 1.0 to 2.0 cm, and 4 kits for larger nodules (Figure 1). Treatment can be performed in outpatient surgery by an operator, a nurse, and an anaesthesiologist and requires about 30-45 min (from targeting to final US assessment).

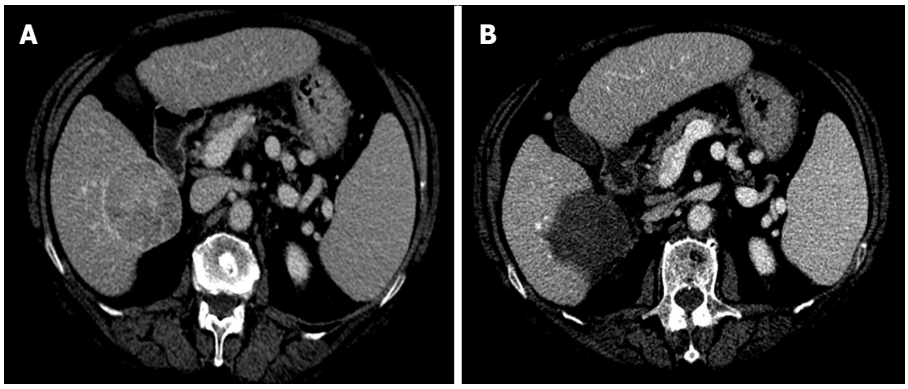

Figure 1 Representative case of complete ablation of large nodule with multi-fibre technique. A: Computed tomography (CT) scan before laser ablation (LA) session shows a nodular lesion 6 cm in maximum diameter (hepatocellular carcinoma moderately differentiated) localized in the S6 with exophytic growth (exophytic component > 40%); B: CT scan performed 4 wk after LA procedure shows complete necrosis of the tumor. Four illuminations were performed using the pullback technique and the treatment lasted 24 min. The procedure was well tolerated and the patient was discharged from the hospital 24 h after the procedure. The only side effects were mild pain and self-limiting fever lasting for 7 d.

TRANSLATING ALL THE AFOREMENTIONED INTO CLINICAL PRACTICE: TOWARDS A PATIENT-TAILORED APPROACH

Before commenting on the role that laser technology plays in percutaneous ablation of HCC, we should briefly summarize the recommended indications commonly accepted by the scientific community so far. On the basis of published data, RFA is now the first-line ablative technique whenever possible[76-81]. PEI has less local control effectiveness but still has a role in achieving complete response when the residual untreated viable tissue is minimal or when location at risk-sites implies serious adverse events or severe complications[82-85]. For solitary HCC ≤ 2 cm, RFA should be considered the first-line treatment for its lower mortality and morbidity, shorter hospitalization, and lower costs (compared to surgery), and should be preferred to PEI due to its greater effectiveness and predictability of treatment results[86]. Survival outcomes of patients with HCC < 3 cm treated by percutaneous approach are competitive with those of surgery. However, a careful multidisciplinary evaluation of the age and comorbidities of the patients and of the location of these tumours is needed[82,84]. In HCC > 3 cm resection or combined treatment (TACE + RFA or PEI) has been suggested to improve survival[87,88], but available studies do not yet provide useful conclusions as the enrollment criteria of patients was too stringent[88]. Studies are needed to define which population can benefit from the combined treatments.

As RFA effectiveness is size-dependent, to obtain complete necrosis the upper limits must not exceed 2.5-3.0 cm[89,90]. To overcome this limitation and obtain larger volumes of necrosis, a variety of devices[91,92] of different shapes and designs[93,94] used either with different algorithms[95] or activated in different modes (consecutive, simultaneous, or switching) has been developed[96-98]. In the treatment of large HCC (≥ 5 cm), conventional RFA is limited mainly by incomplete ablation, with reported complete ablation rate of 74% after single session in lesions between 3 and 5 cm and of 62% in tumours > 5 cm after multiple sessions[99]. Using three internally cooled bipolar electrodes complete, ablation rates was 81% in patients with large HCC[100].

Therefore, multiple heat sources are needed to obtain large volumes of necrosis; the laser technique with multiple thin needle fibres and simultaneous approach[42] satisfies this need. Indeed, LA obtains interesting results with thin, very simple devices that are much less sophisticated and less expensive than those used by RFA. According to the size and shape of the lesions, one to four fibers are used. Two laser fibers for nodules ≤ 2.0 cm and four fibers with tips arranged in a square configuration for larger nodules are used. For a single illumination, laser light is employed for 4-6 min. For nodules > 3.0 cm, multiple illuminations and the pullback technique are employed. The introduction of the novel needle guide has made it possible to obtain a complete ablation of lesions up to 5 cm[43]. No specific methods are used for treating lesions in high-risk (*i.e.*, near gallbladder, main biliary duct, hepatic hilum, adjacent hollow viscera, or exophytic location) and/or hard-to-reach locations (*e.g.*, in the dome of the liver, in the caudate lobe)[42,43,65]. Additionally, this technique makes it is relatively easy to obtain a safety margin ≥ 5 mm in a higher percentage of cases (62%)[53] than that reported by other authors with RFA[101-105]. Furthermore, thin devices makes it possible to treat multiple lesions of the liver of different sizes and in different locations in the same LA session without increasing the complications rate[43]. Therefore, it is possible to customize the ablative treatment according to the size and location of the lesion to be treated. Laser techniques can be used effectively in patients with very early and early HCC (BCLC 0 and A) because of their high percentage of complete response. The reported local effectiveness and long-term outcomes obtained with LA are comparable with those of RFA. Specifically, in the subgroup of Child's class A cirrhotic patients with lesions ≤ 2 cm (BCLC 0-A)

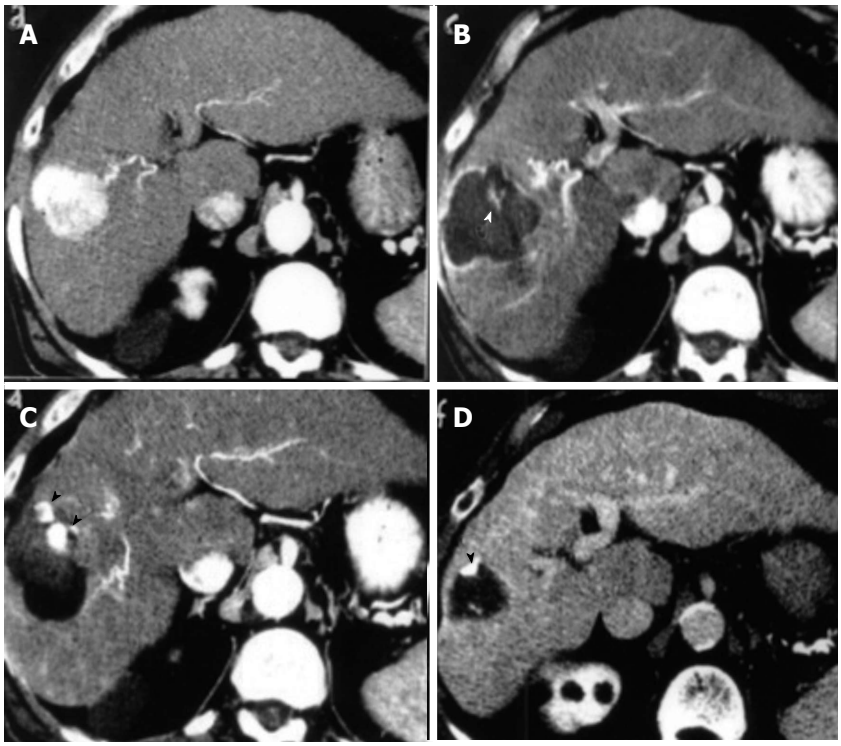

Figure 2 Representative case of complete ablation of hepatocellular carcinoma of 5 cm with combined treatment (laser ablation followed by trans-arterial-chemo-embolization). A: Computed tomography (CT) scan before Laser ablation (LA) shows a lesion 5 cm in diameter beneath the capsule in S8 during arterial phase; B: CT scan after LA shows an area of necrosis larger than basal lesion with small viable foci (white arrowhead) within the zone of coagulation; C: CT scan shows compact retention of iodized oil in the residual viable tissue (black arrowheads) after trans-arterial-chemo-embolization (TACE) session; D: CT scan shows marked volume reduction of treated area and clear shrinkage of viable tissue (black arrowhead) 6 mo after the combined procedure.

without contraindications to surgery treated by LA, 5-year survival was equivalent to that of RFA[68,78] as reported above. Finally, thanks to thin needles and to the more effective tumoricidal action of heat compared to ethanol, we believe that this technique could replace PEI in the treatment of nodules at high-risk sites when RFA is not technically feasible, as has been recommended by some authors[106].

As for the water-cooled laser applicators, it must be emphasized that their main advantage is their MRI compatibility, which allows pre-procedure planning and intra-procedure treatment monitoring using a variety of temperature-sensitive techniques[107,108]. The Frankfurt group has provided compelling long-term survival data in patients treated with this method for the ablation of hepatic metastases[109] and has recently published two papers on primary liver lesions in cirrhotic patients with a high percentage of complete response and low local recurrence[44] (Table 1). However, to achieve these excellent results, the authors used a large cross-sectional probe diameter (3 mm) that requires large bore cannula (9 gauge) for percutaneous treatment. In addition, the diffusion of MRI-guided LA is restricted by machine availability and by complexity of the procedure, requiring between 60 and 120 min to be completed[110,111]. New MRI-compatible applicators permit the execution of the whole procedure within the MR suite, reducing the procedural time and increasing technical effectiveness[112]. However, we think that although interventional MRI guidance is undoubtedly more accurate than US for monitoring ablation, its use would greatly limit the number of centers capable of performing tumor ablation, with ablation procedures being relegated to only those facilities with such specialized equipment. Thus, given that US is readily available, its use has proven to be successful on a practical level in these last 20 years, compared to the potential benefits of less available technologies. In short, these data show that touted advantages of a particular system do not have equal weight in the clinical scenario. Last but not least, we must add the costs of this option to its overall complexity.

A new ablation laser system consisting of 980-nm diode laser with a power of 15-W and diffuser-tipped optical fiber inserted through a 17-gauge internally cooled catheter was recently introduced in field practice. This system achieves a large, well-circumscribed ellipsoid ablation zone up to 2.0 cm × 2.3 cm in a single application lasting about three minutes, and up to 3.7 cm × 3.2 cm with two parallel applicators placed 1.5 cm apart[60]. Due to its characteristics, this system has been applied thus far to focal malignant lesions of the prostate and the brain[24,59]; research and clinical applications on hepatic focal lesions are underway (oral communication). Therefore, the limitations of the previous system, which used high-calibre devices, can be overcome by this technical solution. Further, the execution time of the entire manoeuvre can be shortened significantly by using real-time RM guidance.

Again, *ex vivo* and *in vivo* studies are underway (unpub-

lished data) using diffuser-tipped optical fiber that can be placed in the target area through flexible internally cooled catheter under US guidance. It is possible to produce areas of necrosis of about 3.5 cm × 3.0 cm× 3.0 cm in diameter in about 20 min. If these data are confirmed by clinical studies, we will have made good use of the advantages of US guidance in combination with those deriving from a caliber similar to that of RFA- and MWA-cooled electrode. Therefore, with laser technical improvements such as the new small cylindrical diffuser[60] or the novel needle guide system[43], it possible to employ an array of applicators to increase the ablation zone without increasing invasiveness, procedural complexity, times of ablation, or costs. In clinical practice, a trade-off must be made between these multiple factors and the operator's skill, the available technology, and the biology of the tumor.

While the reported outcome data with combined treatment (LA plus TACE) are interesting, they were obtained with a technique that is the opposite[73] of what is commonly used in referral centers. When surgery is unfeasible, a combined/sequential approach (PEI plus RFA, TACE plus PEI, RFA or MW) should be considered on an individual basis for multinodular nodules and for nodules > 3 cm, after multidisciplinary evaluation[85]. Recently, a meta-analysis of RFA following TACE reported no significant difference in survival rates between RFA plus TACE and RFA for small HCC. On the contrary, this sequential treatment improved overall survival rate in patients with intermediate and large HCC[113]. Therefore, the main indication of combined therapies is for lesions > 3 cm and < 8 cm. Both the PEI and TACE with different mechanism cause a reduction of the blood flow through the tumor, thereby facilitating a larger ablation zone.

LA before TACE, instead, reduces the tumor burden and brings the lesion back within the range of TACE effectiveness. In other words, LA results in a minimal amount of tumor tissue, which can be destroyed with selective TACE using a lesser amount of embolizing material (Figure 2). Because it is possible to destroy lesions up to 5-6 cm with laser technique (Figure 1) we think that this combined method might be effective in treating lesions larger than 6 cm both in cirrhotic patients and in non-cirrhotic patients, thereby avoiding surgery, as currently suggested by some authors[114].

The safety of the procedure was investigated in a multicenter study sufficiently representative both of the type and of the number of possible complications when using either multiple thin needles[75] or large water-cooled devices[44]. The data reported above compare favourably with those of the tested and much more widely used RFA technique and with those of the MWA technique. The mortality rates of RFA range from 0% to 1.5% of cases and major complications from 1.5% to 5.8% of cases[69,115-118]. The mortality and major complications rates of MWA have been reported as 0% to 5.1% and 2.6% to 5.1%, respectively[119-122].

Finally, a few words regarding the MWA technique: its safety profile appears good, but there is still no confirmation on large series of cirrhotic patients. In the only comparative study with RFA, MWA showed comparable therapeutic efficacy and complications rates than RFA, but required more treatment sessions. Furthermore, adequate clinical data are lacking[123].

CONCLUSION

Given that there is not a single method available that meets all the requirements of an ideal ablation system, based on what has been discussed above and on data from the vast literature available, we can reasonably draw some conclusions: (1) the differences between the techniques in terms of the results are modest; (2) one technique may be more difficult than another and more rapid than another. In other words, there are differences in the ease and duration of the various procedures; and (3) while some energy sources may be better suited to certain applications, none has proven suitable for all applications. The laser technique developed in the United Kingdom has been used in the last two decades mainly in German and in Italy but has not been commercialized and sponsored in the rest of the world[124,125]. We hope that in the future a greater availability of the applicators will facilitate their use in clinical practice. The technique has been sufficiently tested and the recent RCT trial should validate it. The fine needle technique offers maximum flexibility, thereby allowing a tailored approach to the characteristics of each nodule in any location of each patient. More in general, we think that the reference centres that treat more than 50 patients/year should be equipped with all the available techniques so as to be able to use the best and the most suitable for each type of lesion for each patient.

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REVIEW

Targeting the insulin-like growth factor pathway in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Only 30%-40% of the patients with HCC are eligible for curative treatments, which include surgical resection as the first option, liver transplantation and percutaneous ablation. Unfortunately, there is a high frequency of tumor recurrence after surgical resection and most HCC seem resistant to conventional chemotherapy and radiotherapy. Sorafenib, a multi-tyrosine kinase inhibitor, is the only chemotherapeutic option for patients with advanced hepatocellular carcinoma. Patients treated with Sorafenib have a significant increase in overall survival of about three months. Therefore, there is an urgent need to develop alternative treatments. Due to its role in cell growth and development, the insulin-like growth factor system is commonly deregulated in many cancers. Indeed, the insulin-like growth factor (IGF) axis has recently emerged as a potential target for hepatocellular carcinoma treatment. To this aim, several inhibitors of the pathway have been developed such as monoclonal antibodies, small molecules, antisense oligonucleotides or small interfering RNAs. However recent studies suggest that, unlike most tumors, HCC development requires increased signaling through insulin growth factor Ⅱ rather than insulin growth factor Ⅰ. This may have great implications in the future treatment of HCC. This review summarizes the role of the IGF axis in liver carcinogenesis and the current status of the strategies designed to target the IGF-Ⅰ signaling pathway for hepatocellular carcinoma treatment.

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Key words: Hepatocellular carcinoma; Insulin; Insulin-like growth factor; Insulin-like growth factor receptor; Therapy; Tyrosine kinase inhibitor; Antibody therapy

Core tip: It is mandatory to develop alternative therapies for the successful treatment of hepatocellular carcinoma (HCC). One of the key drivers of hepatocarcinogenesis is the insulin-like growth factor (IGF) system. Therefore, several inhibitors of this pathway have been developed and their therapeutic potential is being tested in patients with HCC. However, recent studies suggest that IGF-Ⅱ, a member of the pathway, may be more relevant for hepatocarcinogenesis than its close homologue IGF-Ⅰ. The purpose of this review is to summarize these facts within a detailed description of the IGF axis and the alterations of the pathway that lead to HCC. The strategies designed to target the IGF-Ⅰ signaling pathway for HCC treatment are also reviewed.

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INSULIN GROWTH FACTOR SYSTEM

The insulin-like growth factor system is formed by three ligands, three receptors and at least six high affinity binding proteins that work cooperatively to regulate cellular metabolism, proliferation, differentiation and apoptosis in most cells[1]. The three ligands are insulin, insulin growth factor (IGF)- I and -Ⅱ. Each has the highest affinity for a specific receptor named after itself: insulin receptor (IR), IGF- I receptor (IGF-IR) and IGF-Ⅱ receptor (IGF-ⅡR). Furthermore, the IGF axis is composed of IGF high affinity binding proteins (IGFBPs) and IGFBP proteases[2].

IGF- I and IGF-Ⅱ are single chain polypeptides, approximately 7 kDa in size, that share 62% of the amino acid sequence[3]. They were first named somatomedins, because they mediate the activity of growth hormone (GH), also named somatotropin[4]. Later, they were renamed to highlight their similarity with insulin[5]. Insulin is a small hormone secreted by pancreatic beta-cells that maintains normal glucose levels in blood by regulating carbohydrate, protein and fat metabolism. Several excellent reviews on insulin and the insulin pathway have been published recently and therefore, this ligand will not be dealt with great detail in this article[6-8]. IGF ligands are bound with high affinity to IGFBPs. IGFBPs regulate the half-life and bioavailability of IGF- I and IGF-Ⅱ and modulate their accessibility to the receptor[9]. IGFBP activities are closely regulated by post-translational modifications and IGFBP proteases. Most IGFBPs also have functions unrelated to the IGF system[10].

Most of the intracellular activity of IGF- I and IGF-Ⅱ is mediated by the tyrosine kinase IGF-IR whereas insulin exerts its biologic functions mainly through IR[11]. These receptors are homologous because they derive from a common ancestor gene[12]. Despite the fact that IR and IGF-IR share most of their downstream mediators, it has been commonly accepted that IGF-IR activation promotes proliferation and differentiation and IR activation promotes metabolic signaling[11]. Surprisingly, the IGF-ⅡR differs largely from IR and IGF-IR, and sequesters IGF-Ⅱ to internalize it for degradation[13].

IGF-I

IGF- I is the main mediator of GH function in normal embryonic development and postnatal growth[14]. GH is produced and secreted by the pituitary gland to induce body growth[15]. GH binds to the GH receptor in the liver and activates a signaling pathway that leads to transcription of several genes, including IGF- I [16]. Human IGF-I gene can be transcribed from two alternative promoters[17-19]. Furthermore, different mature IGF- I transcripts are produced by alternative splicing and polyadenylation[17,19-22]. These transcripts encode for different preproteins that undergo post-translational modifications and mature by proteolytic cleavage at both ends[23], resulting in a single polypeptide of 70 amino acids (7.5 kDa) cross-linked by 3 disulfide bonds[24,25]. Currently, the impact on IGF- I functionality of such a complex mRNA and protein processing is unclear.

IGF- I is produced by several tissues, including the liver, bone, muscle and brain[26]. The IGF- I produced in these organs acts locally, with the exception of the liver, which produces most of the secreted hormone[27]. Hepatocytes are the main producers of IGF- I in the liver while non-parenchymal cells make a minimal contribution[28]. Liver secretion is possible because IGF- I is not sequestered by liver IGF-IR, which is almost undetectable in healthy hepatocytes[29], and it is only expressed in the liver in non-abundant non-parenchymal cells such as Kupffer cells, hepatic stellate cells (HSCs) and myofibroblasts[28]. Circulating IGF- I levels increase from birth to puberty when they reach their maximum value and then decline with age thereafter[30]. When circulating IGF- I increases, it inhibits the synthesis of GH and IGF- I production is then controlled by negative feedback[31].

IGF- I has similar functions to insulin, since both regulate glucose uptake and their production is affected by nutritional status. IGF- I exerts its function by binding with high affinity to its principal receptor, IGF-IR (Figure 1). However, it can also bind to IR with 100-fold less affinity[32]. IGF- I binding to IGF-IR promotes anabolic processes such as DNA, RNA, protein and glycogen synthesis and results in proliferative and differentiating effects[33].

IGF-II

Unlike IGF- I , IGF-Ⅱ expression is not regulated by GH[34]. In fact, the main regulator of IGF-Ⅱ transcription is still unknown. The *IGF-II* gene is generally an imprinted gene expressed only from the paternal allele[35,36]. However, in the liver this control is only maintained at the fetal stage, as IGF-Ⅱ expression becomes biallelic in the adult liver[37]. This is not due to a real loss of imprinting but to the activation of a biallelic adult liver specific promoter responsible for producing 50% of liver IGF-Ⅱ[38]. The standard imprinted IGF-Ⅱ promoters are still active in the adult liver and account for the remaining expression of IGF-Ⅱ from the paternal allele[37,39]. The *IGF-II* gene encodes a pre-pro-IGF-Ⅱ protein of 180 amino acids that transforms to a 156 amino acid-long pro-IGF-Ⅱ upon peptide signal loss[40]. Most of the pro-IGF-Ⅱ is cleaved and glycosylated to yield the 67 amino acid-long mature IGF-Ⅱ[41].

IGF-Ⅱ can be produced by several tissues, but most comes from both parenchymal and non-parenchymal liver cells[28]. IGF-Ⅱ expression reaches maximal values during the fetal stage, as IGF-Ⅱ plays a crucial role in fetal development[42]. After birth, IGF-Ⅱ levels decrease to 400-600 ng/mL (about 4-fold higher than IGF- I) and remain constant for the rest of life[43]. Despite the higher amount of IGF-Ⅱ than IGF- I , the function of IGF-Ⅱ is gradually replaced by IGF I after birth[2]. Similar to IGF- I , IGF-Ⅱ is able to bind with high affinity to IGF-IR, to regulate cell proliferation and differentiation (see below)[34]. Furthermore, IGF-Ⅱ can bind to IGF-ⅡR, which induces IGF-Ⅱ internalization and degradation[44]. Finally, IGF-Ⅱ can also bind to insulin receptor subtype

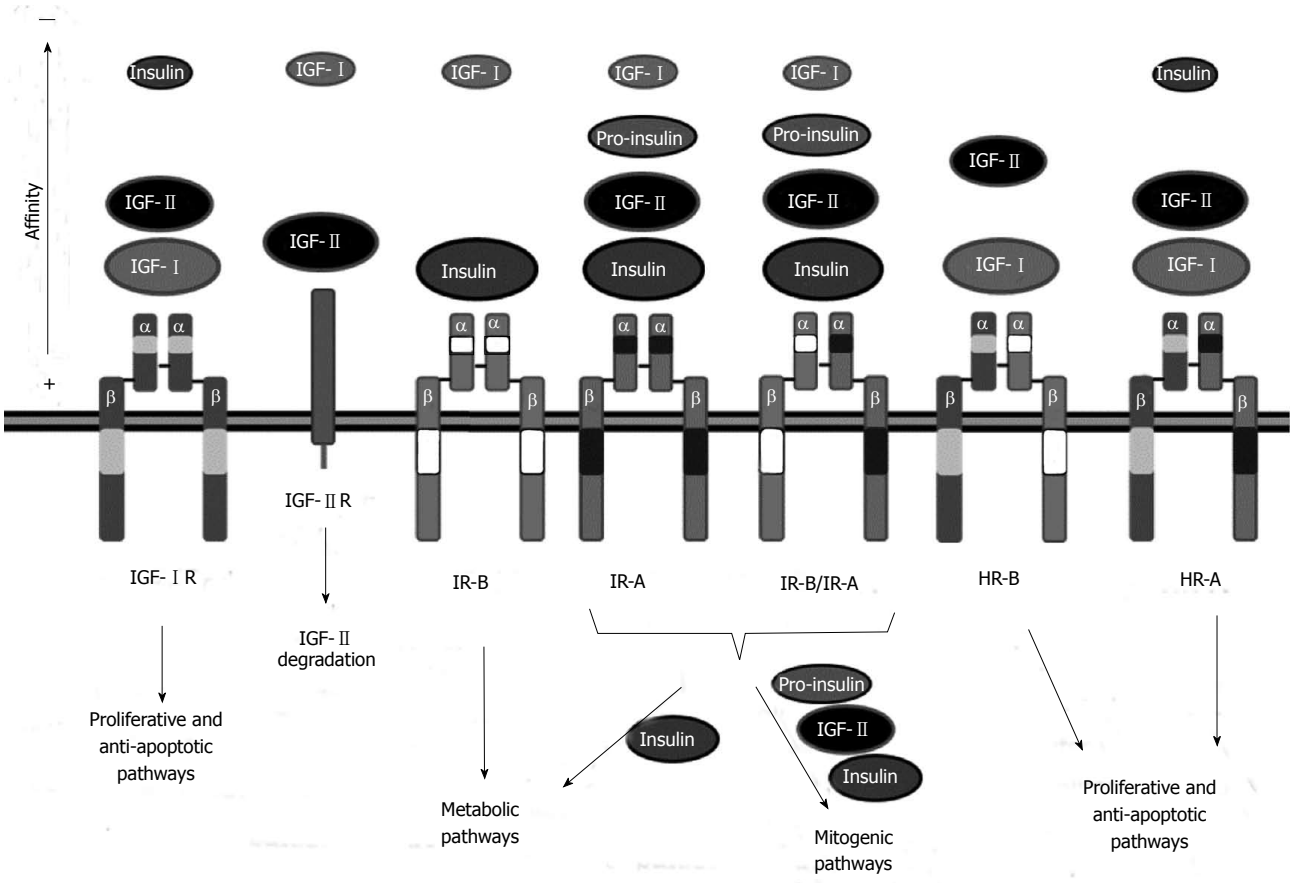

Figure 1 Insulin-like growth factor receptors and their ligands. The insulin-like growth factor (IGF) system is composed of three receptors: IGF-IR, IGF-ⅡR and insulin receptor (IR). IGF-IR is the main receptor of the IGF system. It can bind IGF-Ⅰ and IGF-Ⅱ with high affinity. IGF-ⅡR is a negative regulator of the pathway that binds IGF-Ⅱ and promotes IGF-Ⅱ degradation. Finally, IR mediates insulin signaling. Two IR isoforms exist: the adult IR-B isoform that binds insulin and the fetal IR-A isoform, that can bind IGF-Ⅱ in addition to insulin promoting mitogenic signaling. The IR-A isoform is commonly overexpressed in hepatocellular carcinoma (HCC). IR isoforms can form IR-A/IR-B hybrids which behave as IR-A receptors. Moreover, HR-A or HR-B hybrid receptors can be formed between IGF-IR and IR-A or IR-B respectively. These hybrid receptors lack high affinity binding to insulin and act, similar to the IGF-IR receptor, promoting proliferation and survival.

A (IR-A) to display mainly mitogenic effects[45,46]. Interestingly, both IR-A and IGF-Ⅱ are upregulated in several tumors[47].

Insulin

Synthesis and secretion of insulin is mainly regulated by glucose levels, but other stimuli can also influence these processes[6]. Upon binding to the IR, insulin plays a key role in maintaining normal glucose levels in blood by regulating carbohydrate, protein and fat metabolism[48]. While IR activation after insulin binding promotes mainly metabolic events, recent evidence supports the hypothesis that IR can also mediate mitogenic effects[49,50].

IGFBPs and IGFBP proteases

Six high affinity IGFBPs (IGFBP1-6) have been described which share 36% homology[51,52]. There are other IGFBP-related proteins (designated IGFBP-rP1-10) which bind IGF-Ⅰ and IGF-Ⅱ with lower affinity than classical IGFBPs[52]. IGFBP structure is composed of three domains. The N-terminal and the cysteine rich C-terminal domains are involved in IGF ligand binding and are common to all IGFBPs. The intermediate domain is different for each IGFBP and is probably involved in IGF-independent functions[10]. IGFBPs transcription is cell specific and is tightly regulated by hormones and by growth factors[2]. IGFBP levels are also controlled post-transcriptionally by proteolysis. There are three types of IGFBP proteases: serine proteinases, matrix-metalloproteinases (MMPs) and aspartyl proteinases[30]. IGFBP proteases are relatively specific for each IGFBP because the site of cleavage is inside the hyper-variable domain of the IGFBPs.

IGFBPs are widely expressed, but each tissue preferentially produces one or two classes[10]. The principal source of IGFBPs is the liver. There, hepatocytes express IGFBP1, 2 and 4 while non-parenchymal cells express IGFBP3[28]. After tissue secretion, IGFBPs circulate in blood and extravascular fluids and all of them bind IGF-Ⅰ and IGF-Ⅱ ligands with high affinity (10^{-10} M)[10]. IGFBP2, 5 and 6 have a special preference for IGF-Ⅱ[53]. Interestingly, IGFBPs do not bind insulin because the specific amino acids that confer IGF binding affinity are not conserved in the insulin sequence[25,54]. Ninety-nine percent of circulating IGF-Ⅰ is bound by IGFBPs[2]. This high efficiency of IGF-Ⅰ binding is due to the excess of IGFBPs (50 times higher than IGF-Ⅰ) and to the high binding affinity[55]. Note that the affinity of IGFBPs for IGF ligands is similar or even higher than the affinity of IGF ligands for

their receptors[52].

IGF binding to IGFBPs increases ligand half-life but decreases IGF availability for signaling through IGF receptors. Both IGF-Ⅰ and IGF-Ⅱ are able to form binary complexes of approximately 50 kDa with IGFBPs or ternary complexes of approximately 150 kDa with IGFBP3 (or IGFBP5 to a lesser extend) and the acid-labile subunit (ALS) protein[9]. Almost 75% of the bound IGF forms ternary complexes[56]. When bound to IGFBP3, IGF-Ⅰ half-life increases from 8 to 30 min and when bound to IGFBP3 and ALS, IGF-Ⅰ half-life increases from 30 min to 15 h[57]. However, the IGF-I-IGFBP3-ALS ternary complex is too large to pass through the vascular endothelium to reach the IGF-IR[58]. Therefore, plasmatic proteases are required to break tertiary into binary complexes, able to cross the vasculature. Subsequent proteolysis of IGFBPs by plasmatic or tissue specific proteases releases IGFs and allows IGF signaling[55].

In general, it can be considered that IGFBP1, 3 and 5 activate IGF signaling while IGFBP2, 4 and 6 are inhibitory. However, the same IGFBP can potentiate or inhibit IGF signaling depending on post-translational modifications or binding to other factors[10,59-61].

IGF-IR

IGF-IR is a transmembrane tyrosine kinase receptor expressed ubiquitously. The mature receptor is composed of 2 homodimers, *i.e.*, two α and two β subunits, cross-linked by disulfide bridges (Figure 1). The α subunit (130-135 kDa) is located extracellularly and contains the IGF binding domain, while the β subunit (90-97 kDa) crosses the membrane and reaches the cytoplasm where the tyrosine kinase domain is located[62].

IGF-1R is mainly activated by IGF-Ⅰ. However, it can also bind IGF-Ⅱ and insulin with 2-5 fold and 100-1000 fold less affinity, respectively[63] (Figure 1). Following ligand binding, IGF-IR suffers a conformational change that activates the tyrosine kinase domain, leading to autophosphorylation of specific tyrosines and recruitment of specific docking proteins, including insulin-receptor substrate proteins (IRS-1 to -4) and Shc[64]. Thus, different signaling cascades are activated (Figure 2): (1) IRS-1 phosphorylation activates the phosphatidylinositol-3 kinase (PI3K)-AKT-mTOR pathway that leads to increased glucose uptake and protein synthesis, cell survival and apoptosis inhibition[65]. Following IRS-1 phosphorylation, PI3K is activated by phosphorylation, leading to activation of AKT/PKB[66]. AKT/PKB inhibits apoptosis by activating by phosphorylation anti-apoptotic proteins such as Bcl2, Bclx and NFκB, and by inhibiting by phosphorylation pro-apoptotic proteins such as the Bcl-2 family member Bad, members of the fork head transcription factor (FOXO) family, Fas ligand (FasL) and caspase 9[62,67]. Furthermore, AKT/PKB induces glucose uptake and glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK-3β) activity[68] by phosphorylation of the serine 9 residue[69,70]. Finally, AKT/PKB phosphorylates the DNA repair protein p21. Phosphorylated p21 does not bind PCNA leading to cell cycle progression[71]; and (2) Phosphorylation of the Shc protein activates the RAS-RAF-ERK pathway, related to cell differentiation, proliferation and migration[72]. Phosphorylated Shc complexed with Grb2 and SOS proteins leads to the activation of RAS[73]. RAS induces ERKs, which in turn inhibit apoptosis in a similar way to AKT and induces cell proliferation and migration[74].

Besides these major pathways, IGF-IR may also: (1) activate p38 mitogen-activated protein kinase (p38MAPK), leading to cellular growth and differentiation[75]; (2) bind apoptotic signal-regulated kinase 1 (ASK1), which impedes c-Jun N-terminal kinase (JNK) activation, that in turn, inhibits the apoptosis mediated by death-inducing receptors[76]; (3) lead to the 14-3-3-dependent mitochondrial translocation of Raf, maintaining the mitochondrial integrity, and thus protecting cells from apoptosis[77]; phosphorylate IRS-2 which influences integrin expression, that, together with the IRS-1-dependent decreased cell-cell contact, potentiates cell motility and anchorage independent growth[78]; and (4) affect JAK/STAT-3-mediated inhibition of apoptosis[79]. Thus, in summary, IGF-IR activation leads to differentiation or to increased cell proliferation and migration.

IGF-ⅡR

The human *IGF-ⅡR* gene is imprinted in rodents, where it shows maternal expression[80,81]. Surprisingly, expression in humans is polymorphic: most humans are biallelic but some show imprinted expression[82,83]. *IGF-ⅡR* gene expression results in a transmembrane protein of 2491 amino acids located in the Golgi apparatus (approximately 90%) and in the cell surface (approximately 10%)[84]. The extracellular domain consists of 15 homologous tandem repeats, able to bind with different affinities to mannose 6-phosphate (M6P)-containing proteins or M6P free factors[85]. M6P factors that bind IGF-ⅡR include leukemia inhibitory factor, cathepsin D and latent TGF. M6P free proteins bound by the receptor are urokinase-type plasminogen activator receptor (uPAR), retinoic acid and IGF-Ⅱ[86]. It has been shown that IGF-Ⅰ can also bind to IGF-ⅡR but with very low affinity, while insulin does not bind at all[87] (Figure 1).

The main function of IGF-ⅡR is to transport extracellular and Golgi derived-acid hydrolases and other ligands to lysosomes[86]. Upon IGF-Ⅱ binding, the entire complex is internalized in clathrin-coated vesicles that travel to the endosomal compartment, where the ligand is degraded and the receptor is recycled to the cell membrane[88]. Therefore, in the IGF axis, IGF-ⅡR acts as a scavenger receptor lacking intrinsic signaling. Thus, several studies have demonstrated that IGF-ⅡR may act as a tumor suppressor gene[80,89,90].

Interestingly, some authors have described that IGF-ⅡR may be cleaved from the cell membrane to act as a truncated soluble form of 270-280 kDa[91]. This soluble receptor is detected at very low concentrations (0.1 nmol/L) in the serum and other fluids of several mammalian species[86]. However, it efficiently sequesters circulating IGF-Ⅱ[92].

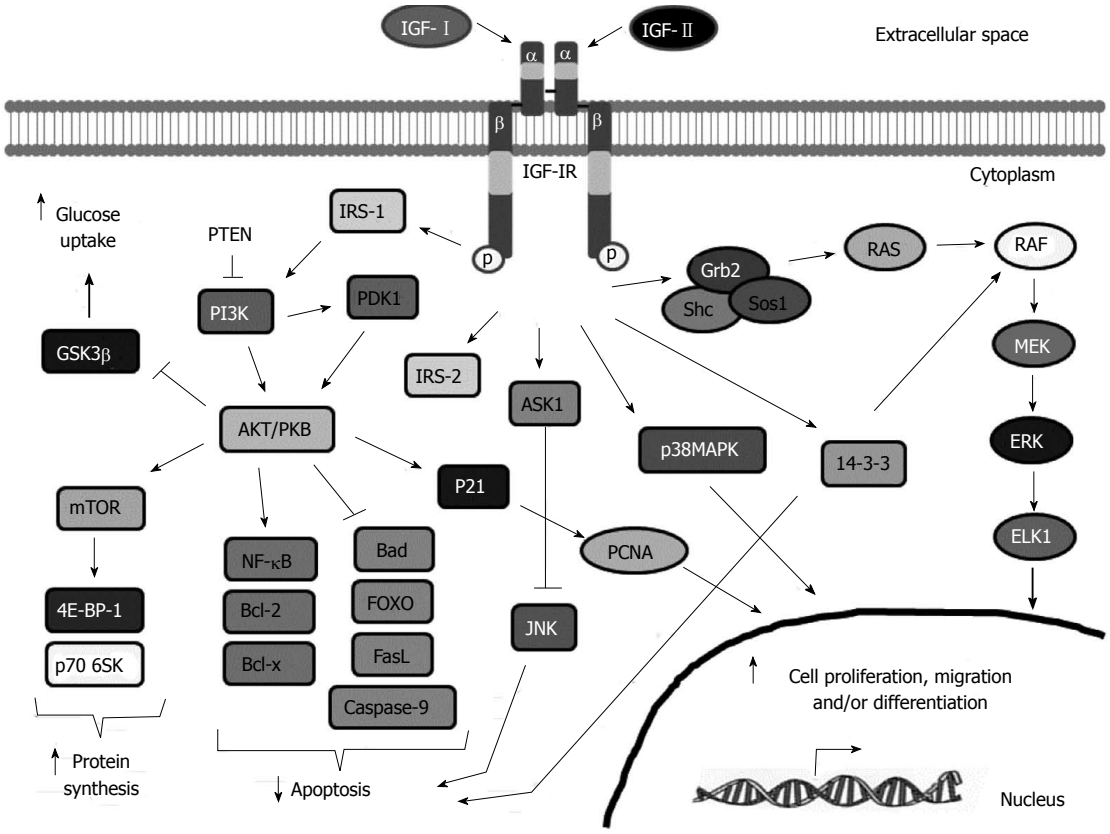

Figure 2 Insulin-like growth factor-I receptor signaling pathway. Insulin-like growth factor- I (IGF- I), IGF- II and to a lesser extent, insulin, can bind to IGF-IR and promote a conformational change that leads to the autophosphorylation and activation of the IGF-IR. Phosphorylated receptor recruits specific docking proteins including IRS1-4, Shc and 14-3-3 which trigger mainly the PI3K/AKT/mTOR and the RAF/MEK/ERK signaling pathways. Activation of the IGF-IR induces protein and glycogen synthesis, glucose uptake, cell proliferation, migration and survival and cell differentiation depending on the cell type. See text for more details. GSK-3β: Glycogen synthase kinase-3; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; PI3K: Phosphatidylinositide 3-kinase; AKT/PKB: Protein kinase B; NFKB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Bcl-2: B-cell lymphoma 2; IRS-2: Insulin receptor substrate 2; FOXO: Forkhead box protein O1; PCNA: Proliferating cell nuclear antigen; RAF: Rapidly Accelerated Fibrosarcoma; MEK: Mitogen-Activated Protein Kinase; ERK: Extracellular-signal-regulated kinase; ELK1: ETS-Like kinase; ELK1: ETS-Like kinase.

IR

IR and IGF-IR share almost the same signaling pathway. Insulin binding to the IR induces a conformational change that results in the autophosphorylation of the IR and the recruitment of IRS proteins or Shc, leading, respectively, to the activation of PI3K-AKT-mTOR and metabolic effects or to the activation of RAS-RAF-ERK and mitogenic effects[6]. In fact there are two isoforms of the IR. The standard receptor is IR-B, expressed in adult liver, muscle and adipose tissue and involved in binding to insulin to regulate glucose homeostasis[62]. Alternative splicing regulation leads to a mature transcript that encodes for IR-A, which lacks 12 amino acids from exon 11 and is expressed by fetal tissues and some tumors[47]. IR-A can bind to insulin, IGF- II and proinsulin with different affinities to promote mainly proliferation, migration and inhibition of apoptosis, but also possess some metabolic activating capacity[62,93] (Figure 1). Insulin binding to IR-A seems to induce more metabolic effects than IGF- II binding[94,95]. IR-A binds insulin with 1.5 fold higher affinity than IR-B and possesses a higher dissociation and internalization rate[95]. Therefore, in cells with increased IR-A:IR-B ratios, most insulin signals through IR-A[7]. This is in line with the higher risk of HCC when insulin serum levels increase[96]. Proliferation can also result from IR-A binding to IGF- II , which interacts with 3-10 fold lower affinity than insulin, or to proinsulin[97].

IR-A and IR-B can form IR-A/IR-B heterodimers that behave similarly to IR-A[7]. It seems that insulin and IGF- II can bind IR-A/IR-B hybrids with the same affinity as IR-A homodimers (Figure 1). IGF- I also binds IR-A/IR-B hybrids with lower affinity[98]. When IR-A is overexpressed, most IR-B forms IR-A/IR-B hybrids, leading to decreased metabolic signaling and increased proliferation[7].

IGF-IR/IR hybrid receptors

Due to the high homology between IGF-IR and IR, IR can also form heterodimers or hybrid receptors (HR) with IGF-IR[99,100]. The cellular content of HR depends only on the molar concentrations of each receptor because IGF-IR/IR heterodimers and homodimers are formed with similar efficiency[101]. Depending on the IR isoform, HR can be IGF-IR/IR-A (HR-A) or IGF-IR/IR-B (HR-B)[7]. HR-A is activated by IGF- I , IGF- II and, to a lesser extent, by insulin, while HR-B is activated mainly by IGF- I but also by IGF- II with lower affin-

ity[102] (Figure 1). Functionally, HRs behave more like IGF- I R than like IR[48].

It is still unclear how the activation of the different IR isoforms, IGF-IR and HR by insulin, IGF- I and IGF- II leads to different biological effects despite the fact that they share most downstream mediators. Differences in ligand binding, internalization or dissociation rates, protein structure and the presence of cell or tissue specific factors could explain this phenomenon[7,11].

HEPATOCELLULAR CARCINOMA

Given its role in cell proliferation, the IGF system is one of the pathways deregulated in cancer. As IGF- I protein is highly expressed in the liver, hepatocellular carcinoma (HCC) has been traditionally linked with increased IGF activity. HCC is the third cause of cancer-related deaths worldwide[103]. The most relevant risk factors in the development of HCC are those that induce liver cirrhosis, as 90% of HCC develops in a cirrhotic liver[104]. Liver cirrhosis is the result of chronic liver disease, due mainly to prolonged alcohol abuse, genetic predisposition, obesity and viral infections with HBV and HCV[105]. These agents induce chronic inflammation that leads to the death of hepatocytes and the activation of hepatic stellate cells (HSCs)[106]. Activated HSCs secrete collagen leading to liver fibrosis and ultimately, the breakdown of liver architecture and functionality. Hepatocyte death and proliferation results in the formation of regeneration nodules, characteristic of the cirrhosis stage[107]. The inflammation coupled with the high proliferation level of cirrhotic hepatocytes leads to the accumulation of mutations and to the loss of epigenetic control that may result in HCC initiation and progression[108]. The genetic and epigenetic events that lead to HCC include somatic mutations, telomere shortening, changes in gene expression profiles and RNA editing and genomic alterations[109,110]. These alterations result in a deregulation of several signaling pathways including PI3K/AKT/mTOR, RAS/RAF/MAPK, WNT, HGF/c-MET, EGFR, IGF-IR and PDGF, leading to hepatocarcinogenesis[103,111]. In the next section, the involvement of the IGF system in the development of HCC will be dealt with in detail. The contribution of other signaling pathways or the IGFI-R activated factors PI3K/AKT/mTOR and RAS/RAF/MAPK has been extensively reviewed by other authors and will not be described in this review[111-116].

Once an HCC is diagnosed, surgical resection is the primary curative treatment followed by liver transplantation and percutaneous ablation[117]. However, only 30%-40% of patients are eligible for these treatments. Moreover, there is a high frequency of tumor recurrence after surgical resection[118]. Unfortunately, most HCC seem resistant to conventional chemotherapy and radiotherapy[119]. The poor efficacy of antitumor agents is also due, at least in part, to the inefficient drug delivery and metabolism exerted by the cirrhotic liver that host the tumor[109]. In the clinical trials searching for alternative therapies for HCC, patients may suffer from unbearable drug toxicity and the treatment must be withdrawn leading to the therapeutic failure of compounds that are promising for the treatment of other tumors.

Thus, the development of novel therapies against HCC is urgently required. To this aim, the identification of oncogenic addiction loops or primary "gatekeeper" and "driver" mutations that would allow for HCC initiation and progression, respectively, is mandatory[109,111]. Furthermore, better therapeutic responses could be obtained after a correct patient stratification. Under the name of HCC there are tumors with different etiologies and tumors generated in response to a broad spectrum of deregulated pathways. Therefore, different HCC may respond differently to different therapies. There is a great need for establishing accurate HCC classifications not only for prognostic purposes but also to select the best therapeutic option for each HCC subtype.

To date, Sorafenib is the only drug approved by the FDA available for patients with advanced HCC. Sorafenib is a multikinase inhibitor that blocks PDGFR, VEGFR and RAF phosphorylation[120] resulting in decreased cell proliferation, activation of apoptosis and inhibition of angiogenesis[121]. In patients with advanced HCC, Sorafenib administration produces a statistically significant increase in the overall survival and a decrease in the time to progression of the disease[122]. Many other agents for HCC treatment are under development. Some drugs such as Sunitinib and Brivanib, showed negative results in phase III trials, as first-line or second-line therapies, respectively[109]. Other agents such as Tivantinib, a c-Met inhibitor against the HGF pathway, have shown promising results in patients with HCC[123]. Tivantinib, is particularly efficient in those HCC with high c-Met expression levels[124], highlighting the need for performing personalized medicine with proper HCC molecular analysis to aid in the choice of successful therapies. The combination of different therapies can also increase success. In fact, Sorafenib used in combination with other techniques or other molecules had synergistic effects in preclinical and clinical models of HCC[121,125]. In this review we will focus on therapies related to the IGF system, as other authors have recently reviewed therapies that affect different signaling pathways[114,125-127].

IGF SYSTEM ALTERATIONS IN HEPATOCARCINOGENESIS

The IGF axis is one of the most commonly deregulated signaling pathways that contribute to cancer development. Alterations have been found in almost all members of the pathway. Here, we review the most important alterations that have been associated with hepatocarcinogenesis.

IGF- I

IGF- I is a mayor ligand of the IGF pathway, highly expressed in the liver and highly protumorigenic for several cancers. Surprisingly, several experiments suggest that

IGF-I expression may be antitumorigenic in the case of HCC. Several results support this hypothesis: (1) In situations of chronic liver damage and functional insufficiency, such as liver cirrhosis, the secretion of liver derived molecules including IGF-I is reduced or even totally suppressed in the most severe cases[128]. As the cirrhotic liver is the substrate for HCC development, decreased IGF-I levels could contribute to hepatocarcinogenesis. In fact, in patients with chronic hepatitis, decreased levels of IGF-I are associated with HCC incidence[129]; (2) Patients with HCC also display lower levels of circulating IGF-I when compared with healthy controls[130]. In fact, the development of HCC is preceded by a significant reduction in IGF-I levels, independently of the degree of impairment of liver function. Thus, a precocious diagnosis of HCC could be performed based on a decrease in serum IGF-I levels[129]. Furthermore, transcriptome analysis reveals that IGF-I mRNA levels are decreased in HCC human samples compared to matching adjacent tissue[131]. This can also be observed when liver tumors develop in mouse models after a single exposure to DEN hepatotoxic. In this case, mouse HCC is induced in a non-cirrhotic liver; and (3) decreased levels of IGF-I are associated with higher tumor invasiveness and poor prognosis[132]. The combination of low IGF-I and high VEGF predicts median overall survival of 2.7 mo compared with 19 mo for patients with higher IGF-I and lower VEGF. Serum IGF1 levels also predict tumour progression and overall survival in patients with HCC who undergo transarterial chemoembolization[133]. Also, the lack of liver IGF-I mRNA increases the risk of HCC recurrence after curative resection[134,135].

IGF-Ⅱ

Excessive IGF-IR signaling is a characteristic feature of liver tumors. Since IGF-I levels are reduced in most HCC, the ligand of the pathway should be insulin or IGF-Ⅱ. In fact, overexpression of IGF-Ⅱ has been estimated to occur in 16%-40% of human HCC[136]. Furthermore, in both *in vivo* and *in vitro* models of HCC, IGF-Ⅱ overexpression correlates with higher cell proliferation[137,138] while IGF-Ⅱ inhibition promotes apoptosis and decreases cellular proliferation[139,140]. Accordingly, miR-615-5p, a miRNA that targets IGF-Ⅱ expression directly, induces a decrease in proliferation and migration of HuH7 and HepG2 human hepatoma cell lines[141]. In patients with HCC, increased intratumoral IGF-Ⅱ mRNA levels are associated with higher metastatic potential whereas increased serum IGF-Ⅱ levels correlate with the presence of extrahepatic metastasis[142,143].

Overexpression of IGF-Ⅱ has been shown to be the result of increased transcription[143]. As IGF-Ⅱ is required for fetal growth, it is expressed mainly during development by the potent paternally imprinted P3 promoter[144]. After birth, transcription of liver IGF-Ⅱ is gradually shifted from initiation at the imprinted promoter to initiation at a biallelic less active P1 promoter. This maintains low levels of liver IGF-Ⅱ throughout adulthood[144]. However, alteration in IGF-Ⅱ imprinting has been described in many tumors[36,145-147]. In HCC, 50%-90% of human biopsies analyzed show a gain of IGF-Ⅱ imprinting[37,148]. This imprinted phenotype results in increased transcription of IGF-Ⅱ from the P3 promoter and decreased transcription from the P1 promoter by hypermethylation resulting in IGF-Ⅱ overexpression[144,149,150]. Furthermore, IGF-Ⅱ hypomethylation at exon 8-9 is found in 90% of HCV-cirrhotic patients analyzed and correlates with higher risk of developing HCC[151].

Other factors may also lead to IGF-Ⅱ overexpression, such as Aflatoxin B1 (AFB1), a potent hepatocarcinogen present in food in developing countries[152]. The tumorigenic effect of AFB1 seems mediated by tumor suppressor genes such as p53 and by an overactive IGF signaling due to overexpression of IGF-IR and IGF-Ⅱ[153,154]. Interestingly, p53mt249, a p53 mutant produced after AFB1 administration, can increase IGF-Ⅱ transcription[154]. Also, the IGF-Ⅱ polymorphism +3580AA, has been associated with higher serum levels of IGF-Ⅱ and has been recently linked to higher risk of HCC in humans[51,155].

Insulin

Little is known about the role of insulin in HCC development. It has been reported that increased insulin serum levels are associated with higher risk of cirrhosis[156] and HCC[96].

IGFBPs and IGFBP proteases

In general, IGFBPs limit bioavailability of IGF ligands, attenuating IGF-IR signaling. Thus, some IGFBPs exert antiproliferative effects in human hepatocarcinoma cell lines. The addition of IGFBP3 to the HepG2 hepatoma cell line is able to counteract the mitogenic effect induced by administration of exogenous IGF-I [157]. Similarly, the administration of IGFBP1-4 results in decreased PLC cell proliferation[158]. Accordingly, the expression of antiproliferative IGFBPs such as IGFBP1, 3 and 4, is downregulated in human HCC[159].

The levels of IGFBP3 are also reduced in cirrhotic patients, but not as much as in HCC samples. Unfortunately, IGFBP3 levels are unable to distinguish between different HCC stages[160]. ALS, which forms a trimeric complex with IGF-I and IGFBP3 incapable of passing through the vasculature and activating IGF-IR[161], has been recently found to be downregulated in human HCC due to genomic loss and hypermethylation[162]. Downregulation of IGFBP3 in human HCC samples has also been linked to promoter hypermethylation[163]. On the other hand, p53, a potent antiproliferative protein, increases the secretion of IGFBP3[164].

IGFBP-rP1, also known as IGFBP7, is a low affinity IGFBP that has been recently identified as a tumor suppressor gene in HCC[13]. Expression of IGFBP-rP1 is dramatically downregulated by astrocyte elevated gene-1 (AEG-1), a novel oncogene that is overexpressed in 90% of the HCC analyzed[165]. In some patients, there is a complete deletion of the *IGFBP-rP1* gene[166]. In others, silencing of IGFBP-rP1 may result from promoter

methylation, which might be used as a biomarker for HCC diagnosis[167]. When IGFBP-rP1 is overexpressed in human HCC cells or tumors, cell growth is inhibited. Interestingly, an inverse correlation between IGFBP-rP1 expression and HCC stage has been found[166].

However, not all IGFBPs display antitumor effects in HCC. Some IGFBPs, such as IGFBP2 and 5, are associated with IGF activation. Accordingly, elevated levels of IGFBP2 have been reported in HCC patients[168]. There are no data on IGFBP5 levels in patients with HCC but inhibition of IGFBP-5 expression exerts antiproliferative effects in the Huh-7 hepatoma cell line[169]. Similarly, as IGFBP proteases release IGF ligands from IGF-IGFBP complexes leading to overactivation of the IGF pathway, they contribute to HCC development. Therefore, increased plasma levels of Cathepsin D, an acidic protease that degrades IGFBP3, have been found in cirrhotic and HCC patients[170]. Moreover, TIMP-1, an inhibitor of MMPs, displays antitumor effects by inhibiting IGFBP3 degradation and IGF-Ⅱ bioavailability[171].

IGF-IR

Signaling through IGF-IR plays an important role in tumorigenesis because of its ability to promote proliferation, protect from apoptosis and potentiate cell migration[29]. IGF-IR overactivation is one of the hallmarks of HCC and can be mediated by increased levels of IGF-IR protein and/or excess of IGF ligands[172]. Healthy mature hepatocytes do not express IGF-IR. In liver cirrhosis the situation is unclear as some authors report that IGF-IR is upregulated while others claim it is downregulated[173]. Most hepatoma cell lines express detectable levels of IGF-IR mRNA and protein[28]. In HCC samples, upregulation of IGF-IR is one of the most common alterations occurring in 30% of the patients[174].

Expression of several downstream components of IGF-IR has been found altered in some HCC samples. IRS-1, the main substrate of IGF-IR activation, is implicated in hepatocarcinogenesis. In fact, 90% of HCC overexpress IRS-1 and IRS-1 overexpression correlates with tumor growth[175]. IRS-2 is also deregulated in HCC. Upregulation of IRS-2 has been found in early and late stages of hepatocarcinogenesis. IRS-2 and IRS-1 have overlapping and specific functions[176]. They are co-overexpressed in 76% of HCC samples and overexpressed independently in 24%[177]. Some studies suggest that a high IRS2/IRS1 ratio may correlate with tumor aggressiveness. In fact, it has been shown that AFB1 increases the levels of IRS-2 but decreases the levels of IRS-1, leading to increased cell migration[178].

IGF-ⅡR

Overactivation of IGF-IR signaling by excessive IGF-Ⅱ molecules can be counteracted by IGF-ⅡR, which decreases IGF-Ⅱ levels through lysosomal degradation[13]. In fact, increased levels of IGF-Ⅱ may result from decreased expression of IGF-ⅡR. Indeed, tumor suppressor characteristics have been attributed to IGF-ⅡR in several tumor types. This has been the subject of a recent review[179]. Thus, inhibition of IGF-ⅡR expression increases cellular proliferation both *in vitro* and *in vivo*[180,181]. Conversely, overexpression of full length IGF-ⅡR into IGF-ⅡR deficient cells decreases cell growth and increases apoptosis *in vitro*[182,183] and decreases tumor growth *in vivo*[183,184]. However, overexpression of IGF-ⅡR has also been associated with an increase in cell number[185]. This is not surprising. It should be taken into consideration that IGF-Ⅱ is not the only ligand for IGF-ⅡR. Overexpression of IGF-ⅡR may affect the signaling of other relevant molecules, such as TGFβ, resulting in proliferative effects[179]. Antiproliferative effects of overexpressed IGF-ⅡR may only occur in cell lines or tumors whose increased proliferation depends on increased IGF-Ⅱ levels.

Given its role as a tumor suppressor protein, IGF-ⅡR is usually found downregulated in cancers, including HCC[186]. Low levels of IGF-ⅡR in HCC result from different alterations such as imprinting, loss of heterozygosity (LOH) and/or mutations[179,180,187-189]. There is a small subset of individuals carrying a paternally imprinted IGF-ⅡR allele[82]. Thus, they only have maternal expression of IGF-ⅡR, as happens with rodents. These individuals, together with rodents, should be more susceptible to developing HCC, as they only require mutations or decrease of gene expression from the active allele to suppress IGF-ⅡR functionality or production[190]. LOH caused by allelic deletion at the 6q26 locus, where the IGF-ⅡR gene locates, has been found in 54.5% of human HCC samples and in a smaller proportion of dysplastic liver lesions[179]. This LOH has also been observed in cirrhotic nodules suggesting that loss of the IGF-ⅡR gene could be an early event in hepatocarcinogenesis[187]. Furthermore, 55% of HCC with IGF-ⅡR LOH present mutations in the remaining allele[189]. Interestingly, while some mutations occur at the IGF-Ⅱ binding site of IGF-ⅡR, most occur in repeats 9 or 10, which are important for M6P-binding[179]. This finding indicates that the binding of M6P-containing proteins to IGF-ⅡR may have antitumoral effects. In fact, the M6P-bearing protein CREG can inhibit cell proliferation by stimulation of lysosomal IGF-Ⅱ degradation[191]. Generally, mutations in IGF-ⅡR lead to the formation of truncated proteins. Interestingly, using a truncated form of IGF-ⅡR derived from a reported splicing mutation[89], it has been demonstrated that truncated proteins can bind IGF-Ⅱ and M6P-containing proteins and are able to form heterodimers with the full length IGF-ⅡR[188]. Surprisingly, these heterodimers are rapidly cleaved and liberated from the cell membrane by MMPs, leading to a great decrease in the amount IGF-ⅡR bound to the cell membrane[188]. These data indicate that truncated proteins can act as dominant negative regulators of IGF-ⅡR contributing to cancer development.

IR

It has recently been published that the IR-A/IR-B expression ratio markedly increases in intratumoral HCC sections but not in adjacent tissues[7]. The relative abun-

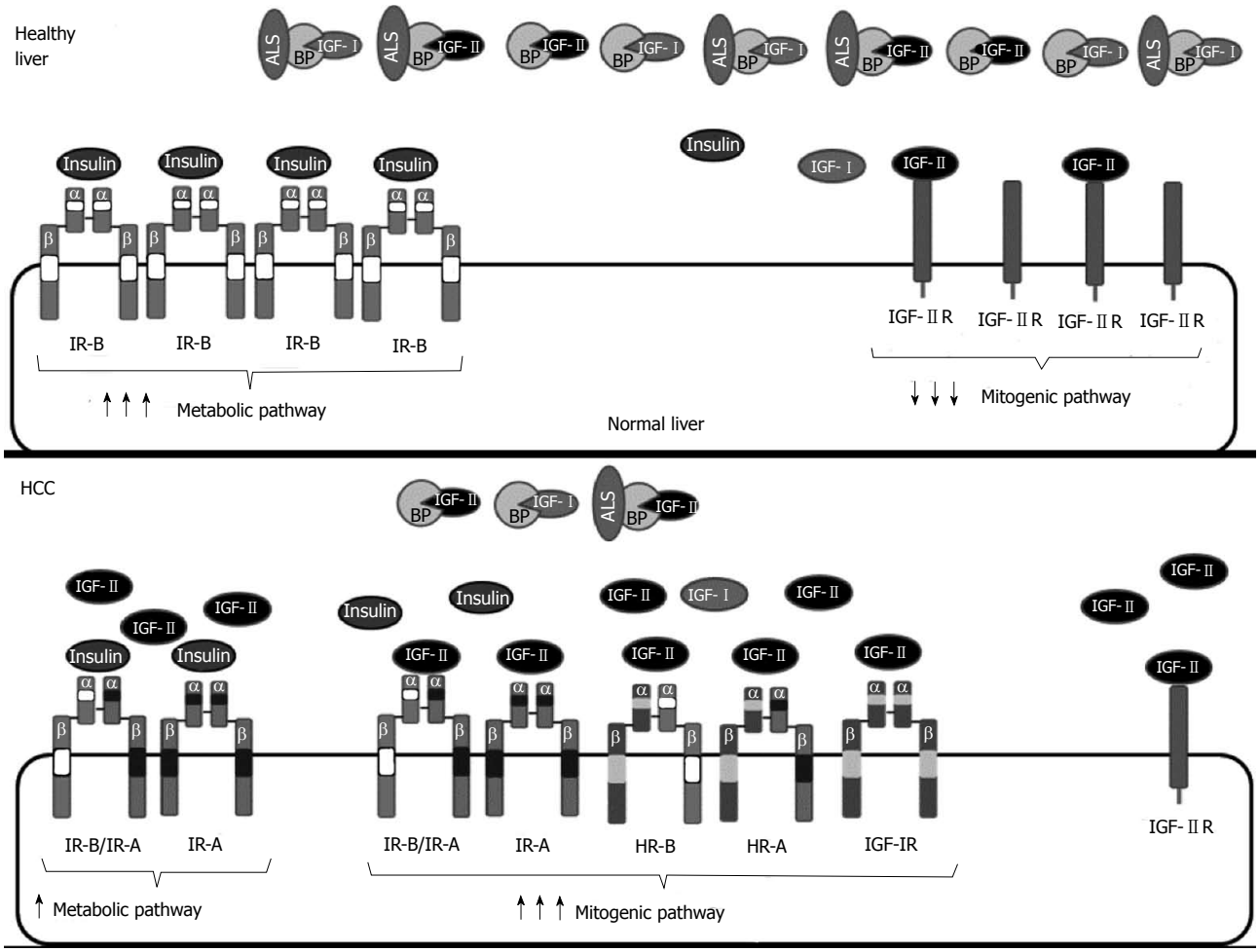

Figure 3 Insulin growth factor system alterations in hepatocellular carcinoma. Healthy hepatocytes secrete high amounts of insulin growth factor (IGF)-Ⅰ and express IGF-ⅡR and IR-B, but do not express IGF-IR. Many HCC are characterized by increased IGF signaling. Such IGF system overactivation is mediated by higher levels of ligands and/or receptors. Levels of functional IGF-Ⅱ increase in many hepatocellular carcinoma (HCC): (1) by reactivation of the fetal promoter of IGF-Ⅱ that leads to IGF-Ⅱ overexpression; (2) by a decrease in circulating IGFBPs that chelate IGF-Ⅱ; or (3) by decreased degradation through IGF-IIR, which is expressed at lower levels due to aberrant imprinting, loss of heterozygosity or gene deletions. Furthermore, IGF-IR and/or IR-A may be overexpressed in HCC. Overexpression of IGF-IR and IR-A leads to an increase in the formation of homodimeric HR-A or heterodimeric IR-A/IR-B receptors that bind IGF-Ⅱ with high affinity resulting in decreased metabolic signaling and increased proliferation.

dance of IR-A, the fetal IR isoform, *vs* total IR mRNAs in normal liver is 5% while in hepatoma cell lines it reaches 50%-75%[192]. The increase of the aberrant splicing that leads to the IR-A isoform is a consequence of the activation of the EGF pathway, one of the most relevant dysregulated pathways in HCC[193]. Interestingly, production of IR-A after EGFR activation only occurs in transformed but not in healthy hepatocytes. High affinity binding of IR-A by IGF-Ⅱ induces mitogenic and anti-apoptotic effects leading to HCC development. Tumors overexpressing both IR-A and IGF-Ⅱ should be resistant to conventional therapies that target IGF-IR.

IGF-IR/IR hybrid receptors and IR-A/IR-B heterodimers

As many HCC overexpress IGF-IR and IR-A and HR formation depends on the concentration of each receptor, these HCC should display an increase in HR-A. Concomitantly, an increase in HR-B and in IR-A/IR-B, which promote proliferation through IGF ligands could also occur together with a decrease in IR-B/IR-B homodimers, responsible for insulin mediated metabolic activity[7]. Interestingly, some cancers have shown an increased fraction of hybrid receptors unrelated with their relative concentrations, suggesting that other factors may be implicated in hybrid receptor formation[194].

In summary, increased proliferation in HCC is generally characterized by an increase in the bioavailability of IGF-Ⅱ, which signals through increased IGF-IR, IR-A homodimers and HR-A leading to increased proliferation and decreased apoptosis (Figure 3). The increase in available IGF-Ⅱ may result from increased *IGF-II* gene expression but also from a decrease of IGF-Ⅱ degradation by IGF-ⅡR or IGF-Ⅱ sequestration by IGFBPs.

TARGETING THE IGF AXIS IN HCC TREATMENT

Compelling evidence exists to involve the IGF system in hepatocarcinogenesis. Therefore, several strategies that target different IGF components are being studied with

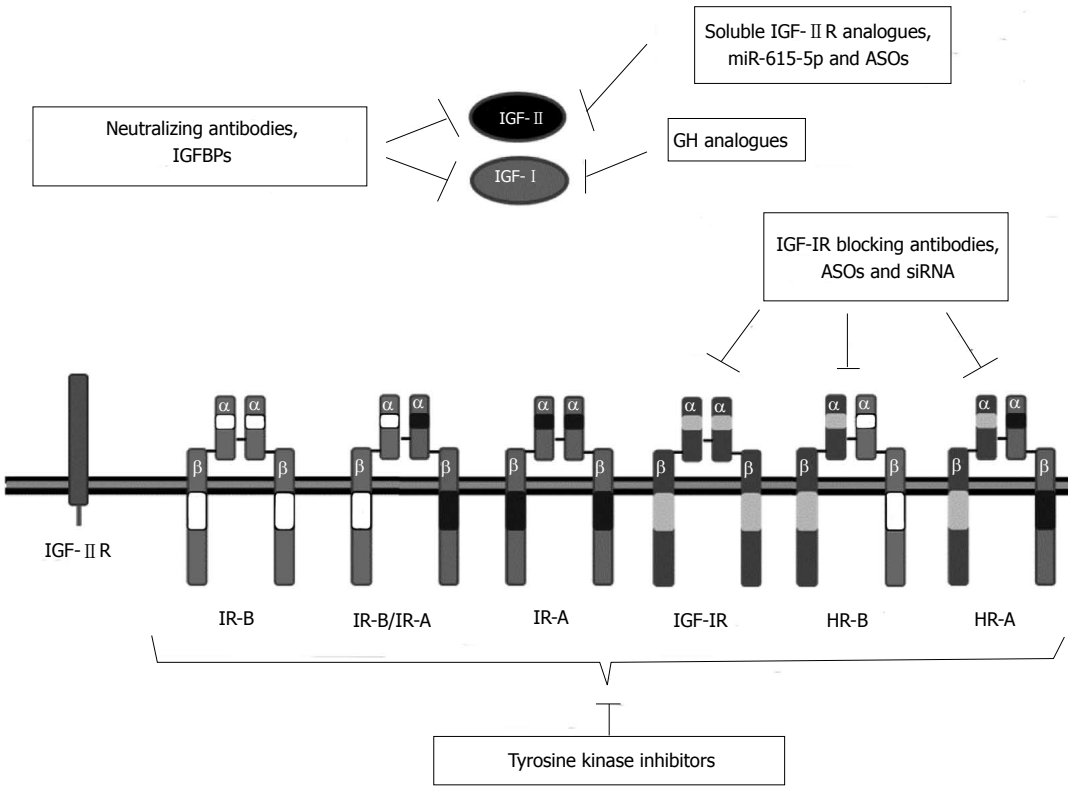

Figure 4 Insulin growth factor targeting strategies in hepatocellular carcinoma treatment. Different strategies can be used to inhibit insulin growth factor (IGF) signaling. They can be ligand based or receptor based therapies. Ligand based therapies affect signaling through all IGF receptors, but these strategies should not be therapeutic in tumors with activating mutations in IGF-IR receptor or downstream proteins. Growth hormone (GH) analogues reduce IGF-Ⅰ expression by blocking GH-GHR interaction. Specific ASOs and miR-615-5p decrease IGF-Ⅱ expression. Soluble forms of IGF-ⅡR and IGFBPs can decrease IGF-Ⅱ bioavailability. Ligand directed neutralizing antibodies impede binding to receptors. Receptor based therapies focus mainly on the inhibition of IGF-IR signaling. Antisense oligonucleotides (ASOs) and siRNAs decrease IGF-IR expression and IGF-IR blocking antibodies impede ligand mediated activation. These strategies do not affect IR-A and IR-A/IR-B, which could be crucial in the progression of some hepatocellular carcinoma. Tyrosine kinase inhibitors inhibit insulin receptor (IR)-A, IGF-IR and hybrid receptor autophosphorylation and subsequent activation but commonly they also inhibit IR-B receptor which is essential for the metabolism of normal cells. IGFBPs: IGF high affinity binding proteins.

the aim of developing new therapeutic drugs for HCC (Table 1)[28]. The most promising strategies include the inhibition of IGF-IR signaling using monoclonal antibodies against IGF-IR, IGF-Ⅱ and/or IGF-Ⅰ and small molecule tyrosine kinase inhibitors (TKIs) that inhibit IGF-IR activation and signaling (Figure 4). Other approaches such as the inhibition of IGF-IR or IGF-Ⅱ expression using siRNAs or antisense oligonucleotides (ASO) and the modulation of the IGFBP activity are under preclinical investigation. Some strategies that target the IGF system may be effective as monotherapies but others may be more effective in combination with chemotherapy and can be used as chemosensitizing agents.

These different strategies should be tested exclusively in HCC cells with an altered IGF-Ⅰ axis. Furthermore, the expression and functionality of the different members of the pathway should be evaluated prior to treatment. This should ensure that the therapy is targeting an IGF pathway molecule relevant for the growth of the particular HCC to be treated. Such personalized medicine is essential to obtain therapeutic effects. To help in this analysis, an antibody array has been recently developed that detects ten members of the IGF system (IGF-Ⅰ, IGF-IR, IGF-Ⅱ, IGF-ⅡR, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP6 and insulin)[195]. Unfortunately, antibodies for IR-A and IR-B isoforms are not included in the array, despite their importance in HCC development.

Targeting IGF-Ⅰ

Despite the capability of IGF-Ⅰ to activate IGF-IR signaling, IGF-Ⅰ is decreased in liver tumors and in the serum of patients with HCC[130,131]. Furthermore, high circulating levels of IGF-Ⅰ are associated with less aggressive HCC, with a better prognosis[132] and with a better outcome in patients with advanced HCC receiving Sorafenib[196]. The reason for this is unclear.

Alternatively, IGF-Ⅰ could lead to increased differentiation in hepatocytes and decreased proliferation. In fact, IGF-Ⅰ has a therapeutic role in human liver cirrhosis, the substrate of HCC development. In a pilot study, the intravenous administration of recombinant IGF-Ⅰ in patients with advanced liver cirrhosis improved liver functionality[197]. Moreover, we have demonstrated that the administration of IGF-Ⅰ from viral vectors before the induction of liver cirrhosis prevents the development of the disease in rat models[198]. Furthermore, administration of viral vectors expressing IGF-Ⅰ into rat cirrhotic livers leads to the complete reversion of liver cirrhosis[198,199].

Table 1 Agents targeting insulin-like growth factor system

Compound	Company	Description	Target	Treatment	Status	Ref./Clinical trial	Unaffected mitogenic IGF proteins/metabolic signaling status
Octreotide	Novartis Farmaceuticals	Somatostatin homologue	IGF-I	Monotherapy	Preclinical	[200,201]	IGF-II and insulin interaction with IGF-IR, IR-A, IR-B/IR-A and HRs/not affected
DX-2647		Neutralizing antibody	IGF-II and proIGF-II	Monotherapy	Preclinical	[202]	Insulin interaction with IR-A, IR-B/IR-A and HR-A/not affected
MEDI-573	AstraZeneca (MedInmune)	Neutralizing antibody	IGF-II and IGF-I	MEDI-573 + Sorafenib	Phase I	NCT01498952; [192,203]	
Cixutumumab (IMC-A12)	ImClone Systems Inc	Blocking antibody	IGF-1R	Monotherapy	Phase II	NCT00639509; [53,215,216]	IGF-I, IGF-II and insulin interaction with IR-A, IR-B/IR-A and HRs/not affected
				Cixutumumab + Sorafenib	Phase I	NCT01008566; NCT00906373	
AVE1642	Sanofi-Aventis	Blocking antibody	IGF-1R	Monotherapy	Phase I	NCT00791544; [125]	
				AVE-1642 + Sorafenib	Phase II	NCT00791544	
				AVE-1642 + Erlotinib	Phase II	NCT00791544	
BIIB022	Biogen-Idec	Blocking antibody	IGF-1R	BIIB022 + Sorafenib	Phase I	NCT00956436; [53,223]	
Figitumumab (CP-751,871)	Pfizer	Blocking antibody	IGF-1R, HRs	Monotherapy	Preclinical	[224,225]	IGF-II and insulin interaction with IR-A, IR-B/IR-A/not affected /impaired.
Linsitinib (OSI-906)	OSI Pharmaceuticals	TKI	IGF-1R, IR	Monotherapy	Phase II	NCT01101906; [226,227]	
				OSI-906 + Sorafenib	Phase II	NCT01334710	
AG1024 (Tyrphostin)		TKI	IGF-1R, IR	Monotherapy	Preclinical	[231]	
NVP-AEW541	Novartis Farmaceuticals	TKI	IGF-1R, IR	Monotherapy	Preclinical	[232,233]	
BMS-536924	Bristol-Myers Squibb	TKI	IGF-1R, IR	Monotherapy	Preclinical	[234]	
GSK1904529A	GlaxoSmithKline	TKI	IGF-1R, IR	Monotherapy	Preclinical	[235]	

IGF: Insulin-like growth factor; IGF-IR: IGF-I receptor; IR: Insulin receptor; TKI: Tyrosine kinase inhibitor.

This therapeutic effect correlates with IGF-I mediated activation of an anti-inflammatory and anti-fibrogenic program. Furthermore, IGF-I expression increases differentiation of cirrhotic hepatocytes and liver functionality. Using our model, overexpression of IGF-I in healthy or cirrhotic livers for more than a year did not lead to detectable liver tumors. Given that IGF-I displays anti-inflammatory and hepatoprotective effects, IGF-I deficiency caused by liver cirrhosis may create an intrahepatic microenvironment that allows for hepatocyte dedifferentiation and facilitates HCC emergence. Alternatively, high IGF-I levels could mark for functional hepatocytes, which are more difficult to transform and easier to cure. Finally, it cannot be ruled out that IGF-I could have an unexpected antitumoral effect on its own. If this is the case, IGF-I and IGF-Ⅱ signalling through IGF-IR should lead to different responses in HCC. This has never been shown experimentally. Therefore, even if IGF-I could exert some antitumoral effect, it would be risky to overexpress IGF-I once a HCC with altered IGF axis has developed.

Even if downregulation of IGF-I together with upregulation of IGF-IR, IR-A and IGF-Ⅱ are common events in HCC, suggesting that IGF-I has a limiting role in hepatocarcinogenesis, there are ligand-based therapies that specifically target IGF-I without affecting IGF-Ⅱ. Theoretically, blocking IGF-I could favor binding of IGF-Ⅱ to IGF-IR and increase cell proliferation. This has not been observed with Octreotide, a cyclic octapeptide used as GH analogue in the treatment of several types of cancers. Octreotide competes with GH for binding to its receptor and inhibits GH-GHR interaction and signaling leading to a decrease in IGF-I synthesis by the liver, but also affecting the expression of many other molecules[200]. Furthermore, decreased IGF-I should result in increased GH levels. Octreotide treatment showed good results in prostate cancer, but not in HCC. In a recent meta-analysis of all randomized controlled clinical trials using Octreotide for HCC patients, there was an improvement in the overall survival of the treated patients compared with non-treated controls, which was not statistically significant when compared with placebo controls[201].

Targeting IGF-Ⅱ

IGF-Ⅱ is overexpressed in human HCC and it activates

proliferation and migration through IGF-IR and IR-A receptors. IGF-Ⅱ based therapies must be designed to decrease or normalize IGF-Ⅱ levels and/or to inhibit its interaction with both receptors without altering insulin signaling. To this aim, blocking antibodies and strategies to decrease IGF-Ⅱ expression are under development.

Human antibody DX-2647 binds to IGF-Ⅱ and pro-IGF-Ⅱ with high affinity impeding their interaction either with IGF-IR and IR-A and suppressing proliferation in several HCC cell lines. Moreover, DX-2647 administration delays tumor growth and inhibits angiogenesis in xenograft models of HCC. DX-2647 also reacts with IGF-Ⅰ but with a 200-fold lower affinity[202]. This antibody remains in a preclinical status. Similarly, human monoclonal antibody MEDI-573 binds IGF-Ⅱ and IGF-Ⅰ (with 150-fold lower affinity than IGF-Ⅱ) without interacting with insulin[203]. MEDI-573 impedes IGF binding to IGF-IR, IR-A, and IGFBP3[192]. MEDI-573 administration reduces proliferation in cells expressing either IGF-IR or IR-A receptors but also in mixed populations of cells expressing both receptors in which an IGF-IR-specific antibody was totally ineffective. These results were obtained in several mouse embryonic fibroblast cell lines overexpressing specific human proteins and were then validated in xenograft tumors[203]. Two phase Ⅰ clinical trials designed to determine the effect of MEDI-573 administration on patients with solid tumors have recently finished but the results have not been yet published. Interestingly, a new clinical trial will test MEDI-573 administration in combination with Sorafenib in unresectable or metastatic HCC.

Strategies to decrease IGF-Ⅱ expression, such as antisense (ASO) or methylated (MONs) oligonucleotides, are under preclinical investigation. ASOs are short single-stranded DNA molecules complementary to a chosen mRNA sequence. ASO binding to the target mRNA results in mRNA expression inhibition as a result of RNase H-mediated mRNA degradation and translation blockage[204]. Downregulation of IGF-Ⅱ expression using ASOs that target IGF-Ⅱ mRNA inhibits cellular growth in hepatoma cell lines, but only in those that overexpress IGF-Ⅱ[205]. MONs that bind to the IGF-Ⅱ P4 promoter results in target DNA methylation and in turn, downregulation of fetal IGF-Ⅱ expression in the Hep3B human hepatoma cell line and in Hep3B derived tumors leading to enhanced survival[140]. Further development will be required to deliver these agents to most tumor cells.

Decreased IGF-Ⅱ availability can also be achieved by increased IGF-ⅡR expression. The administration of the soluble form of IGF-ⅡR (sIGF-ⅡR) to myeloid cell lines leads to a decrease in proliferation and survival[206]. Moreover, IGF-Ⅱ-induced DNA synthesis can be counteracted in hepatocytes and fibroblast using sIGF-ⅡR[92]. However, this soluble receptor can also bind other proteins and may have undesirable side effects. Therefore, therapeutic effects should be evaluated using a soluble form of IGF-ⅡR that only contains the IGF-Ⅱ binding domain.

Targeting IGFBP

IGFBPs modulate IGF signaling by regulation of IGF bioavailability. Most IGFBPs inhibit IGF signaling by limiting ligand access to IGF receptors, with the exception of IGFBP2 and IGFBP5. Therapies based on the administration of inhibitory IGFBPs or the inhibition of activating IGFBPs could be developed. In fact, the effect of increased levels of IGFBP3 and IGFBP-rP1 has already been evaluated.

As IGFBP3 represents 90% of serum IGFBPs[53], IGFBP3 downregulation in cancer significantly increases IGF ligand bioavailability. It has already been shown that the administration of exogenous IGFBP3 inhibits cell proliferation in hepatoma cell lines[157,158]. Interestingly, IGFBP3 expression can be re-induced in liver cancer cells by histone deacetylase inhibitors such as Trichostatin A[207,208]. An ongoing phase Ⅰ clinical trial combines Vorinostat, the first histone deacetylase inhibitor approved by the FDA, with different chemotherapy agents in patients with upper gastrointestinal cancers including liver cancer. Finally, as overexpression of IGFBP-rP1 (IGFBP7) decreases the tumorigenic potential of HCC cell lines[166], the antitumoral properties of an adenovirus expressing IGFBP7 have been recently demonstrated in both in vitro and *in vivo* models of HCC[209].

Targeting IGF-IR and IR/IGF-IR hybrids

Different strategies have been described to block IGF-IR signaling including blocking antibodies, siRNAs, antisense oligonucleotides, small molecule inhibitors and tyrosine kinase inhibitors.

Monoclonal antibodies: The administration of monoclonal antibodies against IGF-IR induces apoptosis and decreases proliferation in HCC[210]. Some of the monoclonal antibodies that have demonstrated promising results in preclinical models are: cixutumumab or IMC-A12 is a human IgG1 monoclonal antibody that selectively binds to IGF-IR, preventing the binding of its natural ligands[211]. The antibody also activates internalization and degradation of IGF-IR, leading to decreased levels of this receptor. Thus, IMC-A12 treatment inhibits downstream signaling in several tumors without altering insulin signaling[212-214]. *In vitro* and *in vivo* studies using different HCC models showed that blockage of IGF-IR by IMC-A12 decreases cell proliferation and increases apoptosis, resulting in prolonged survival and delayed tumor growth[215]. On the basis of these results, a phase Ⅰ clinical trial was performed in patients with advanced solid tumors. However, only partial responses were obtained[53]. In a subsequent phase Ⅱ study, administration of IMC-A12 as monotherapy in patients with advanced HCC displayed no antitumoral activity[216]. Instead, half of the patients developed hyperglycemia and 62% of the patients required initiation or increase in active therapy for diabetes. Besides, several patients showed reduced liver function indicated by elevated transaminases and bilirubin and decreased albumin, suggesting that

by blocking IGF-IR a protective effect of IGF-Ⅰ on liver function had been lost. The mayor outcome of the study is that increased levels of IGFBP-1 correlated with progression free survival and with overall survival. The lack of therapeutic effect of IMC-A12 could be explained by the lack of IGF-IR in most of the patients, as IGF-IR expression could only be demonstrated in HCC samples obtained from 21% of the patients. However, the patients whose tumors were positive for IGF-IR did not show correlation with survival when compared with the IGF-IR-negative patients[216]. It needs to be determined whether IMC-A12 is more effective as a chemosensitizing molecule. Therefore, two clinical trials using combination of IMC-A12 and Sorafenib are ongoing.

AVE1642 is a humanized monoclonal antibody against IGF-IR that inhibits growth and metastasis in different human xenograft tumor models when used alone and/or in combination with chemotherapy[217-221]. AVE1642 was first tested in humans with advanced multiple myeloma yielding good tolerability but insufficient activity[222]. A posterior phase Ⅰ/Ⅱ clinical trial testing AVE1642 alone or in combination with Sorafenib or Erlotinib in patients with advanced or metastatic liver carcinoma supported the safety of AVE1642 in combination with active doses of Sorafenib[125].

BIIB022 is a human non-glycosylated IgG4.P antibody that blocks IGF-Ⅰ and IGF-Ⅱ binding to IGF-IR[223]. Preclinical data suggest that BIIB022 administration inhibits the growth of HepG2-derived tumors without induction of hyperglycemia. As BIIB022 lacks an Fc effector function, it displays less toxicity in normal IGF-IR expressing tissues[53]. A phase Ⅰ study to evaluate the tolerability and safety of combinatorial therapy with Sorafenib has been completed but the results have not yet been published.

CP-751,871, also known as Figitumumab, is a human IgG2 antibody that inhibits IGF-Ⅰ and IGF-Ⅱ mediated autophosphorylation of IGF-IR but not IR, resulting in the internalization of the receptor[224]. It has been tested in 8 HCC cell lines, 2 of which, HepG2 and SNU368, were sensitive to the treatment in a dose-dependent manner. Administration of Figitumumab to HepG2 xenograft tumors leads to substantial growth inhibition[225]. Interestingly, in contrast to the other blocking antibodies, Figitumumab is able to inhibit hybrid receptor signaling. In fact, Figitumumab sensitivity has been associated with the levels of N-linked glycosylated IGF-IR/IR hybrids[225]. This compound has reach a phase III trial in multiple myeloma and non-small cell lung cancer, but it has not yet been tested for liver cancers.

Tyrosine kinase inhibitors: OSI-906 is a dual IGF-1R and IR Tyrosine kinase inhibitors (TKI) that displays antitumoral activity in several human cell lines and xenograft tumor models[226]. The mechanisms that mediate sensitivity to OSI-906 have been tested in a panel of 21 human HCC cell lines. In this study, higher responsiveness to OSI-906 was obtained in cell lines expressing high levels of IGF-Ⅱ and IR[227]. Thus IGF-Ⅱ and IR could be used as predictive markers for sensitivity to OSI-906 in HCC patients. OSI-906 evaluation in phase I dose escalation studies, alone or in combination with anti-cancer agents, resulted in good disease control rates and limited toxicity, including hyperglycemia, nauseas, vomiting and fatigue[8]. Two phase Ⅱ clinical trials testing OSI-906 in patients with HCC have been carried out but were terminated due to the safety issues observed in the phase Ⅰ study or to company policies. The partial results of the trials have not yet been published.

AG1024 (Tyrphostin) is a selective IGF-IR and IR TKI that is currently in preclinical development. Blockage of IGF-IR with AG1024 exerts antiproliferative and pro-apoptotic effects in several cancer cell lines[228-230]. Recently, AG1024 has been tested in two IGF-IR-expressing HCC, resulting in a significant decrease in cell invasiveness and a slight caspase-3 dependent proapoptotic effect[231].

NVP-AEW541 is a novel small molecule inhibitor of the IGF-IR tyrosine kinase activity. NVP-AEW541 has a 26-fold higher affinity for IGF-IR than for IR[232] and induces cell cycle arrest and apoptosis in HCC cell lines without cytotoxicity. When NVP-AEW541 was combined with chemotherapy, an additive antiproliferative effect was observed[233]. The effect of NVP-AEW541 remains to be tested in *in vivo* models of HCC.

BMS-536924 is a novel orally active, ATP-competitive, tyrosine kinase inhibitor of IGF-IR and IR. BMS-536924 antiproliferative activity has recently been described in HCC cell lines[234].

GSK1904529A is a tyrosine kinase inhibitor that blocks IGF-IR and IR phosphorylation. GSK1904529A has been tested in a wide range of cell lines and human xenograft tumor models resulting in low toxicity and strong antiproliferative and antitumoral effects. Although no HCC samples were included, the authors demonstrated that GSK1904529A inhibits the activity of the IR in liver tissues suggesting that it could be also effective in HCC[235].

The design of TKIs that target specifically IGF-IR signaling without altering IR signaling is difficult because of the high homology between these two receptors[62]. On one hand, targeting of both receptors can be advantageous since specific inhibition of IGF-IR was associated with higher IR signaling. On the other hand, targeting IR could lead to altered insulin signaling and unwanted secondary effects.

Antisense oligonucleotides: Phosphorothioate ASOs, which are more resistant to nuclease degradation than unmodified DNA, have been designed to target IGF-IR and have been evaluated in a model of HCC. In this study, inhibition of IGF-IR expression by ASOs results in a significant reduction of HepG2 proliferation. Systemic administration of IGF-IR ASOs in nude mice with orthotopic human HCC xenografts results in reduced tumor growth, recurrence and lung metastasis[236].

CONCLUSION

Deregulation of the IGF system is a common feature in HCC. Recent studies suggest that downregulation of IGF-Ⅰ together with upregulation of IGF-Ⅱ and overactivation of IGF-IR and IR-A are important events in HCC development. Thus, increased IGF-Ⅱ bioavailability, caused by increased IGF-Ⅱ expression or decreased regulation by IGF-ⅡR or IGFBPs, could be responsible for IGF-IR and IR-A overactivation. Furthermore, mutations in factors located downstream IGF receptors, such as Ras, PI3K or PTEN, could induce cell proliferation in tissues with normal IGF ligands or receptors. This has not been the subject of this review. Insulin and IR-B, by coupling to IGF-IR and IR-A, could also play a role in IGF pathway activation that leads to HCC. Little is known about the role of insulin in HCC development. Increased insulin serum levels have been associated with higher risk of HCC. This is probably caused by insulin binding to IR-A homo or heterodimeric receptors.

The role played by IGF-Ⅰ in HCC should be studied in detail. It is unclear why IGF-Ⅰ deregulation seems relevant for hepatocarcinogenesis. Experiments that address the effect of IGF-Ⅰ overexpression or downregulation in HCC development should be performed in animal models. The results may be relevant for the management of HCC in humans. Also, efforts should be devoted to understand why the binding of IGF-Ⅰ, IGF-Ⅱ, or insulin to a specific receptor of the IGF pathway, such as IGF-IR or IR-A/IR-B derived receptors, results in the activation of different signals. It should be interesting to identify liver-specific factors that modify IGF-IR signaling according to the ligand that has been sensed by the receptor, either IGF-Ⅰ or IGF-Ⅱ.

Given the particular features of IGF deregulation in HCC, the most promising therapies to date for HCC are antibodies that block IGF-Ⅱ or IGF-IR and tyrosine kinase inhibitors. The success of the treatment may depend on following personalized medicine protocols that first ensure that the IGF system is deregulated in the HCC to be treated. Furthermore, these protocols should evaluate the serum levels of IGF-Ⅰ, IGF-Ⅱ and insulin and the levels within the tumor of all the IGF ligands, receptors, binding proteins and signaling pathway factors. Such a detailed study of each tumor is essential to decide on a successful therapy. Thus, IGF-IR blocking antibodies are expected to be effective in tumors with increased IGF-IR and poor IR-A activation (Table 1). If this analysis is not performed, functional drugs may show no therapeutic effects. This may be the reason why IGF-IR antibodies display antitumoral effects in preclinical models but only partial responses in clinical trials. In the case of TKIs, as they are able to block IGF-IR and IR-A signaling, they are expected to be effective in all HCC with altered IGF ligands and receptors. However, TKI can also inhibit other tyrosine kinase receptors causing unwanted effects. TKI interaction with IR-B should lead to altered insulin metabolism.

Future therapies that target the IGF system should be developed for the treatment of HCC and other tumors. Novel specific antibodies or small molecules that affect the stability of IGF-Ⅱ or impede IGF-Ⅱ being sensed by IGF-IR and IRs should be developed. Similarly, design of functional TKI inhibitors or other molecules that affect IGF-IR and IR-A, but not IR-B is mandatory. Moreover, expression of key activators of the IGF pathway could be affected by antisense inhibitors or genome editing strategies. This will require the improvement of delivery techniques that allow the efficient delivery of the drugs to most tumor cells. Furthermore, present and future therapies need to take into consideration the altered drug metabolism of cirrhotic livers. As most HCC develop in a cirrhotic liver, it may be useful to stratify the patients according to liver functionality and liver fibrosis status before analyzing the therapeutic effects of a particular drug. Finally, even if the IGF system is altered in many HCC, it is not a unique tumor driver. It will be interesting to analyze the results of successful but also of non-successful trials to address if the blockade of IGF pathway was effective and whether other signaling pathways have been induced for tumor survival upon IGF system blockage. This may lead to rationalized combination therapies that may be essential for the successful treatment of HCC.

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MINIREVIEWS

Lipid-lowering agents in the management of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in developed countries and is associated not only with increased risk for liver disease-related complications but also with higher cardiovascular morbidity. Accordingly, lipid-lowering agents are frequently considered in these patients to reduce cardiovascular risk. However, there have been concerns regarding the safety of these agents in patients with chronic liver diseases. In the present review, we discuss the safety of lipid-lowering agents in patients with NAFLD as well as their effects on both cardiovascular and liver disease in this population. Accumulating data suggest that statins are safe in patients with NAFLD and that they reduce the increased cardiovascular morbidity of this population. However, it is still unclear whether statins are also useful as a treatment for NAFLD *per se*, since there are very limited and conflicting data on their effects on liver histology. There is also very scarce evidence regarding the safety and efficacy of other lipid-lowering agents in patients with NAFLD. Randomized controlled studies are needed to evaluate the role of lipid-lowering agents and particularly statins for the prevention of both cardiovascular and liver disease-related complications in this high-risk population.

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Key words: Nonalcoholic fatty liver disease; Statins; Fibrates; Ezetimibe; Colesevelam; Omega-3 fatty acids; Nicotinic acid; Cardiovascular disease; Transaminases; Nonalcoholic steatohepatitis

Core tip: Accumulating data suggest that statins are safe in patients with nonalcoholic fatty liver disease (NAFLD) and that they reduce the increased cardiovascular morbidity of this population. However, it is still unclear whether statins are also useful as a treatment for NAFLD *per se*, since there are very limited and conflicting data on their effects on liver histology. There is also very scarce evidence regarding the safety and efficacy of other lipid-lowering agents in patients with NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by increased amount of fat in the liver in the absence of increased alcohol consumption[1]. NAFLD covers a wide range of histological disorders, ranging from isolated hepatic steatosis to nonalcoholic steatohepatitis (NASH), which is characterized by the coexistence of steatosis with varying degrees of inflammation and fibrosis, whereas some patients progress further to develop cirrhosis[2,3]. NAFLD is the most common chronic liver disease in developed countries[4-6]. Indeed, 34%-46% of the general population has liver steatosis and 12% has NASH[4,5]. Moreover, almost 75% of patients with persistently elevated transaminase levels have NAFLD[6].

Several cross-sectional studies showed that patients with NAFLD have a greater atherosclerotic burden and a higher prevalence of cardiovascular disease (CVD)[7-9]. Moreover, observational studies suggest that patients with NAFLD have increased cardiovascular risk and that CVD is the leading cause of death in this population[10-13]. Since NAFLD and CVD have many common risk factors (*e.g.*, abdominal obesity, type 2 diabetes mellitus (T2DM), insulin resistance, inflammation and oxidative stress), the increased CVD risk in patients with NAFLD might be partly explained by their shared pathogenesis[14-17]. However, there is increased CVD risk in patients with NAFLD even in the absence of T2DM, suggesting that NAFLD is directly causative of CVD[18].

Given the increased cardiovascular risk of patients with NAFLD, aggressive management of CVD risk factors is an essential part of the treatment of these patients. Lipid-lowering treatment is one of the pillars of CVD prevention strategies and primarily consists of administration of statins aiming at reducing low-density lipoprotein cholesterol (LDL-C) levels. The rationale behind this approach is that elevated LDL-C levels are a major independent cardiovascular risk factor[19] and that LDL-C lowering with statins reduces CVD morbidity and mortality[20]. However, an increase in transaminase levels is the most common adverse effect of statins[21]. Moreover, physicians are reluctant to administer statins in patients with elevated transaminase levels[21]. Similar considerations apply for other lipid-lowering treatments, which can be considered in patients who do not achieve LDL-C levels despite treatment with statins or in patients with elevated non-high density lipoprotein cholesterol (non-HDL-C) levels[22]. On the other hand, preliminary data suggest that statins and other lipid-lowering agents might reduce transaminase levels in patients with NAFLD and might also have beneficial effects on CVD morbidity[23,24].

In the present review, we discuss the safety of lipid-lowering agents in patients with NAFLD as well as their effects on both CVD and liver disease in this population.

STATINS IN PATIENTS WITH NAFLD

Safety

Accumulating data suggest that statins are safe in patients with NAFLD. In an observational study in hyperlipidemic patients with elevated transaminases, the incidence of further increase in transaminase levels during treatment with statins was similar compared with patients who had elevated transaminase levels but were not prescribed a statin[25]. Moreover, the incidence of severe elevations in transaminases did not differ during statin treatment between patients who had elevated transaminase levels at baseline and those who had normal transaminases[25].

Randomized controlled studies also support the safety of statins in patients with NAFLD. The West of Scotland Coronary Prevention Study trial compared the effects on CVD events of pravastatin 40 mg/d and placebo in men without established CVD but with LDL-C levels > 155 mg/dL whereas the Cholesterol and Recurrent Events and Long-term Intervention with Pravastatin in Ischemic Disease trials compared pravastatin 40 mg/d and placebo in patients with established coronary heart disease (CHD). In a post-hoc analysis of these 3 trials, the risk of further increase in transaminase levels among patients who had elevated transaminase levels at baseline was similar in those treated with pravastatin and those administered placebo[26]. In the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) trial, patients with myocardial infarction (MI) were randomly assigned to receive atorvastatin aiming at LDL-C levels < 100 mg/dL or conventional treatment; only 14% of the latter group received a statin. In a post-hoc analysis of this trial, patients with elevated transaminase levels < 3 times the upper limit of normal (ULN) who were given atorvastatin (mean dose 24 mg/d) experienced a normalization of transaminase levels[22]. In contrast, patients with elevated transaminase levels who did not receive statins did not show any change in transaminase levels[23]. Similar results were observed in the Assessing the Treatment Effect in Metabolic Syndrome Without Perceptible Diabetes trial, where treatment of patients with metabolic syndrome with atorvastatin at a mean dose of 24-34 mg/d resulted in normalization of transaminase levels in the subgroup of patients with elevated transaminase levels at baseline[27].

Very recently, a post-hoc analysis of the Incremental Decrease in End Points Through Aggressive Lipid Lowering (IDEAL) trial also showed that treatment of patients with MI with atorvastatin 40-80 mg/d or simvastatin 20-40 mg/d reduces transaminase levels in patients with elevated levels at baseline[24]. It should be emphasized that the diagnosis of NAFLD in all these studies was not based on liver biopsy but on the presence of fatty liver in ultrasound and on the exclusion of other common causes of chronic liver disease (*i.e.*, chronic hepatitis B or C, increased alcohol consumption)[22,23,26]. Moreover, patients with transaminase levels > 3 times the ULN were excluded from all studies[23,24,27].

Based on these reassuring data regarding the safety of statin treatment in patients with elevated transaminase levels, current guidelines state that mild elevations of transaminase levels (< 3 times the ULN) are not a contraindication for the administration of statins, provided that patients are followed-up regularly[1,28]. Importantly, statins do not interact with agents that are used in the treatment of NAFLD (*e.g.*, vitamin E, pioglitazone, metformin, ursodeoxycholic acid, angiotensin receptor blockers) and therefore, can be safely coadministered with the latter agents[1,29].

Effects on cardiovascular events

Emerging data suggest that statins are not only safe in patients with NAFLD but also decrease the elevated CVD risk of this population[30]. In the GREACE trial, treatment with atorvastatin reduced CVD events by 39% compared with no statin treatment in patients with MI and normal transaminase levels at baseline[23]. In contrast, CVD morbidity was reduced by 68% with atorvastatin

treatment in patients with elevated transaminase levels, a reduction significantly greater than in patients with normal transaminase levels[23]. The IDEAL trial recently confirmed these findings. In IDEAL, atorvastatin 40-80 mg/d reduced major CVD events more than simvastatin 20-40 mg/d in patients with MI and elevated transaminase levels[24]. In contrast, the incidence of major CVD events did not differ between atorvastatin- and simvastatin-treated patients with normal transaminase levels[24]. Despite these promising data on the effects of statins on CVD morbidity in patients with NAFLD and the increased CVD risk of this population, it should be emphasized that current guidelines do not differentiate LDL-C targets between patients with NAFLD and the general population[22]. Accordingly, LDL-C targets are < 70 mg/dL in patients with NAFLD who have established CVD, T2DM or chronic kidney disease. In the absence of the latter comorbidities, LDL-C targets are < 70, < 100 and < 115 mg/dL in patients with NAFLD and SCORE risk ⩾ 10, 5-9 and 1%-4%, respectively[22].

Effects on liver histology

There are very limited data on the effects of statins on liver steatosis, inflammation and fibrosis in patients with NAFLD. In small uncontrolled studies (n = 4-22), treatment with statins reduced steatosis and ballooning but had no effect on fibrosis; the effect on inflammation was inconsistent between studies[31-35]. In the only randomized placebo-controlled study, the administration of simvastatin for 12 mo in 16 patients with NASH had no effect on liver histology compared with placebo[36]. The interpretation of the findings of these studies is obviously hampered by the small number of patients and the lack of a control group in most of them. Moreover, the follow-up time might have been too short to evaluate the effects of statins on liver fibrosis. Considerably longer follow-up will also be required to assess any benefit of statins on the long-term sequelae of NAFLD, *i.e.*, cirrhosis and hepatocellular cancer (HCC). Notably, observational studies reported a decreased risk of HCC in patients treated with statins regardless of the cause (NAFLD, hepatitis B or C)[37-39]. Indeed, in a recent meta-analysis of 10 studies (n = 1459417), statins reduced the risk for HCC by 37%[37]. Given the limited data on the effects of statins on liver histology in patients with NAFLD, recent guidelines mention that statins should not be used as a treatment for NAFLD[1].

OTHER LIPID-LOWERING AGENTS IN PATIENTS WITH NAFLD

Ezetimibe

In patients who cannot achieve LDL-C targets despite treatment with the maximal tolerated dose of a potent statin, ezetimibe can be added to statin treatment[22]. Ezetimibe does not appear to be associated with increased risk for transaminase elevations when administered to patients with transaminase levels within the normal range[40]. In an uncontrolled study in 8 patients with NAFLD, treatment with ezetimibe for 1 year reduced transaminase levels but had no effect on liver steatosis assessed with ultrasonography[41]. In another uncontrolled study in 10 patients with NASH, treatment with ezetimibe for 6 mo reduced transaminase levels and ameliorated steatosis in liver biopsy but had no effect on ballooning, inflammation or fibrosis[42]. In another uncontrolled study in 45 patients with NAFLD, ezetimibe reduced transaminase levels and ameliorated steatosis, inflammation and ballooning in liver biopsy but had no effect on fibrosis after 2 years[43]. In a recent randomized controlled study in 32 patients with NAFLD, ezetimibe combined with diet for 6 mo had similar effects on transaminase levels and on liver histology as diet alone[44]. There are no randomized controlled studies that evaluated whether combination of ezetimibe with statins reduces CVD events more than monotherapy with statins.

Bile-acid binding resins

Another option to achieve LDL-C targets in patients who do not reach them despite treatment with the maximal tolerated dose of a potent statin is to add a bile-acid binding resin (BAS)[22]. These agents lack systemic side effects since they are not absorbed by the gastrointestinal tract and are not associated with increases in transaminase levels[45]. Colesevelam is a newer member of this class and is associated with lower rates of gastrointestinal side effects than other BAS[45]. However, in a recent randomized, placebo-controlled study in 50 patients with NASH, treatment with colesevelam for 24 wk increased liver steatosis assessed with magnetic resonance imaging[46]. Nevertheless, in the subgroup of patients who underwent a second liver biopsy at the end of follow-up (n = 31), the effects of colesevelam on liver steatosis, inflammation and fibrosis were similar to those of placebo[46]. An early uncotnrolled study in 10 patients with NASH reported a decrease in transaminase levels, steatosis and inflammation but no change in fibrosis after treatment with another BAS, probucol, for 1 year[47]. In contrast, in a more recent uncotrolled study in 26 patients with NASH, treatment with probucol for 6 mo decreased transaminase levels but had no effect on steatosis, ballooning, inflammation or fibrosis[48]. The Lipid Research Clinics Coronary Primary Prevention Trial is the only study that evaluated the effects of BAS on CVD events and showed that treatment of hypercholesterolemic men without CHD with cholestyramine for 7.4 years reduced CHD events compared with placebo[49]. However, no separate analyses of the effects of cholestyramine on CVD events were performed in patients with elevated transaminase levels.

Fibrates

In patients at high or very high CVD risk who have triglyceride levels > 200 mg/dL after achieving LDL-C targets with a statin, fibrates can be added to achieve non-HDL-C targets[22]. The combination of fenofibrate with statins does not appear to increase transaminase or

creatine kinase levels more than statin monotherapy[50]. In contrast, the combination of gemfibrozil with a statin is associated with increased risk for rhabdomyolysis and is contraindicated[22]. Regarding the effects of fibrates on NAFLD, in a placebo-controlled study in 27 patients with NAFLD, fenofibrate had no effect on hepatic triglyceride content[51]. In a larger study in 186 patients with MetS and NAFLD, the combination of fenofibrate and atorvastatin was not more effective than atorvastatin monotherapy in reducing transaminase levels and liver echogenicity[52]. In the only study that evaluated the effects of fenofibrate on liver histology, the administration of fenofibrate for 48 wk in 16 patients with NAFLD decreased transaminase levels and improved ballooning but had no effect on steatosis, inflammation or fibrosis[53]. The only study that evaluated the effects of fibrate and statin combination on CVD events is the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial[50]. In ACCORD, patients with T2DM who were being treated with simvastatin 20-40 mg/d were randomized to receive fenofibrate or placebo[50]. After a mean follow-up of 4.7 years, CVD event rates did not differ between the 2 groups[50]. Again, there have not been performed separate analyses of the effects of fenofibrate on CVD events in patients with elevated transaminase levels who were enrolled in the ACCORD trial.

Omega-3 fatty acids

Another option to reach non-HDL-C targets is to add omega-3 fatty acids to statin treatment[22]. This combination is not associated with increased risk for transaminase elevations[54]. Small uncontrolled studies in patients with NAFLD reported a reduction in transaminase levels during treatment with omega-3 fatty acids[55,56]. In 2 controlled studies (n = 40 and 144, respectively), omega-3 fatty acids combined with diet reduced transaminase levels and hepatic fatty infiltration in ultrasound more than diet alone in patients with NAFLD[57,58]. In the only study that assessed the effects of omega-3 fatty acids on liver histology, treatment with the omega-3 fatty acid eicosipentanoic acid (EPA) for 12 mo reduced transaminase levels in 23 patients with NASH[59]. An improvement in liver steatosis, ballooning, inflammation and fibrosis was observed in 6 out of 7 patients who underwent liver biopsy at the end of follow-up[59]. The only study that evaluated the effects of high doses of omega-3 fatty acids on CVD events is the Japan EPA Lipid Intervention Study (JELIS), in which Japanese patients with hypercholesterolemia were randomly assigned to receive statin alone or statin combined with EPA 1800 mg/d[54]. The addition of EPA reduced CVD events by 19% compared with statin monotherapy[54]. However, this study was performed in a population with increased background fish consumption and it is unclear whether these findings are applicable to other populations[54]. Again, the effects of omega-3 fatty acid and statin combination on CVD events were not analyzed separately in patients with elevated transaminase levels in the JELIS trial.

Nicotinic acid

A final option to achieve non-HDL-C targets is to combine statins with nicotinic acid[22]. However, this combination is associated with increased risk for elevations in transaminase levels compared with statin monotherapy[60,61]. Moreover, there are very limited data on the effects of nicotinic acid in NAFLD. In a placebo-controlled study in 27 patients with NAFLD, nicotinic acid had no effect on hepatic triglyceride content[51]. More importantly, 2 recent studies showed that the combination of nicotinic acid with a statin does not decrease CVD events more than statin monotherapy[60,61]. In the Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes (AIM-HIGH) study, patients with CVD who were on simvastatin 20-40 mg/d, were randomized to receive nicotinic acid or placebo[60]. After a mean follow-up of 3 years, the incidence of the primary end-point (death from CHD, nonfatal MI, ischemic stroke, hospitalization for an acute coronary syndrome, or symptom-driven coronary or cerebral revascularization) did not differ between the 2 groups and an increase in the risk of ischemic stroke was observed in patients who received nicotinic acid[60]. In the Heart Protection Study 2 - Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) study, patients with established CVD who were on simvastatin 40 mg/d were randomized to receive nicotinic acid or placebo[62]. After a median follow-up of 4 years, the incidence of CVD events did not differ between the two groups[62]. Neither of these studies evaluated separately patients with elevated transaminase levels.

CONCLUSION

Accumulating data suggest that statins are safe in patients with NAFLD and that they reduce the increased cardiovascular morbidity of this population. However, it is still unclear whether statins are also useful as a treatment for NAFLD *per se*, since there are very limited and conflicting data on their effects on liver histology. There is also very scarce evidence regarding the safety and efficacy of other lipid-lowering agents in patients with NAFLD. Randomized controlled studies are needed to evaluate the role of lipid-lowering agents and particularly statins for the prevention of both CVD and liver disease-related complications in this high-risk population.

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MINIREVIEWS

Comprehensive review of post-liver resection surgical complications and a new universal classification and grading system

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Abstract

Liver resection is the gold standard treatment for certain liver tumors such as hepatocellular carcinoma and metastatic liver tumors. Some patients with such tumors already have reduced liver function due to chronic hepatitis, liver cirrhosis, or chemotherapy-associated steatohepatitis before surgery. Therefore, complications due to poor liver function are inevitable after liver resection. Although the mortality rate of liver resection has been reduced to a few percent in recent case series, its overall morbidity rate is reported to range from 4.1% to 47.7%. The large degree of variation in the post-liver resection morbidity rates reported in previous studies might be due to the lack of consensus regarding the definitions and classification of post-liver resection complications. The Clavien-Dindo (CD) classification of post-operative complications is widely accepted internationally. However, it is hard to apply to some major post-liver resection complications because the consensus definitions and grading systems for post-hepatectomy liver failure and bile leakage established by the International Study Group of Liver Surgery are incompatible with the CD classification. Therefore, a unified classification of post-liver resection complications has to be established to allow comparisons between academic reports.

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Key words: Complication; Liver failure; Bile leakage; Renal failure; Ascites; Coagulation disorder; Surgical site infection

Core tip: The large degree of variation in the post-liver resection morbidity rates reported by previous studies might be due to a lack of consensus regarding the definitions and classification of post-liver resection complications. The Clavien-Dindo classification of postoperative complications is widely accepted internationally. However, it is difficult to apply to some major post-liver resection complications. Therefore, a unified classification of post-liver resection complications has to be established to allow comparisons between academic reports.

Ishii M, Mizuguchi T, Harada K, Ota S, Meguro M, Ueki T, Nishidate T, Okita K, Hirata K. Comprehensive review of post-liver resection surgical complications and a new universal classification and grading system. *World J Hepatol* 2014; 6(10): 745-751 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i10/745.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i10.745

INTRODUCTION

Liver resection has become a safe operation, and its mortality rate is now almost zero, which is much lower than the rate seen a decade ago[1-3]. Liver resection is the best curative option for patients with certain types of liver cancer such as hepatocellular carcinoma[4,5] and metastatic liver cancer[6], as it is cost effective and results in a shorter period of disease-related suffering. To reduce the invasiveness of surgery, laparoscopic procedures have been widely adopted for various types of liver resection[2,7-9]. Preliminary clinical studies have demonstrated that compared with open surgery laparoscopic liver resection results in fewer surgical complications, less intraoperative bleeding, and shorter hospital stays whilst achieving similar oncological outcomes[2,10].

Although the mortality rates described by previous studies were similar, the reported post-liver resection morbidity rates varied markedly due to the use of different definitions for each complication. In fact, the overall morbidity rate of open liver surgery has been reported to range from 4.1% to 47.7%[2,11]. Dindo *et al*[12] attempted to unify the definitions of post-liver resection surgical complications by developing their own grading system (Table 1), which has been widely accepted according to surgical academic reports. However, a classification of the complications seen after hepatobiliary surgery produced by the International Study Group of Liver Surgery (ISGLS)[13] was incompatible with the definitions outlined in Clavien's classification. For example, cases that involve surgical or radiological interventions performed under general anesthesia (categorized as Ⅲb under the Clavien-Dindo classification) are rarely seen in the clinical setting. Furthermore, patients who suffer organ failure usually exhibit multiple complications, and thus, it is difficult to identify a single cause of the organ failure.

Therefore, we reviewed the definitions of post-liver resection surgical complications and have developed a simple grading and classification system to allow academic reports to be compared.

POST-HEPATECTOMY LIVER FAILURE

Liver failure is the most serious complication after liver resection and can be life-threatening[14,15]. The etiologies of post-hepatectomy liver failure (PHLF) include a small remnant liver[16], vascular flow disturbance[17], bile duct obstruction[15], drug-induced injury[18], viral reactivation[19], and severe septic conditions[15]. In 2011, the ISGLS defined PHLF as a postoperative reduction in the ability of the liver to maintain its synthetic, excretory, and detoxifying functions, which is characterized by an increased international normalized ratio and concomitant hyperbilirubinemia on or after postoperative day 5[13]. Treatments for PHLF must be selected carefully based on the etiology of the condition. Since it was proposed, most reports have employed the ISGLS definitions of PHLF (Table 2). In addition to the latter definitions, our grading system also includes information about the management strategies that are typically employed to treat each PHLF grade (Table 2).

BILE LEAKAGE

Bile leakage (BL) is a major complication of liver resection. The incidence of BL is reported to be 4.0% to 17%[20], and a previous meta-analysis did not find any difference in the incidence of BL between open and laparoscopic cases[21]. BL is defined as an increased bilirubin concentration in the drain or intra-abdominal fluid; *i.e.*, a bilirubin concentration at least 3 times greater than the simultaneously measured serum bilirubin concentration[22]. Once BL develops, it can sometimes lead to complications and can become difficult to manage without interventional radiology (IVR). One of our representative Grade C cases is shown in Figure 1. BL is usually managed with extensive IVR, and reoperations are rarely required. The ISGLS has also developed a grading system for BL[22]. Although the different grades of PHLF are well defined based on clinical symptoms and the management strategies employed, the definitions of each BL grade are too subjective. Therefore, our grading system includes clinical examples (Table 3).

ACUTE RENAL FAILURE

Acute renal failure (ARF) is associated with various postoperative complications. Renal failure is closely associated with PHLF and can lead to hepatorenal syndrome (HRS). The International Ascites Club (IASC) defined HRS using the following criteria[23-25]: (1) cirrhosis and ascites are present; (2) the patient's serum creatinine level is greater than 1.5 mg/dL (or 133 mmol/L); (3) no sustained improvement in the serum creatinine level (to a level of 1.5 mg/dL or less) is seen at least 48 h after diuretic withdrawal and volume expansion with albumin (recommended dose: 1 g/kg body weight per day up to a maximum of 100 g of albumin/d); (4) shock is absent; (5) the patient is not currently taking nor have they recently been taking nephrotoxic drugs; (6) parenchymal kidney disease, as indicated by proteinuria of greater than 500 mg/d, microhematuria (> 50 red blood cells/high power field), and/or abnormal renal ultrasonography, is absent (Verna EC1, Wagener G, Renal interactions in liver dysfunction and failure).

On the other hand, post-liver resection ARF is still poorly defined. Therefore, we have proposed a grading system for post-liver resection ARF (Table 4). The management of ARF mainly involves dehydration and the use of diuretics[26]. Most cases of Grade A and Grade B ARF are reversible and manageable via the latter approach. We defined cases in which the patient could not pass urine without continuous diuretic use as Grade B. On the other hand, Grade C cases were defined as those in which the patient required hemodialysis.

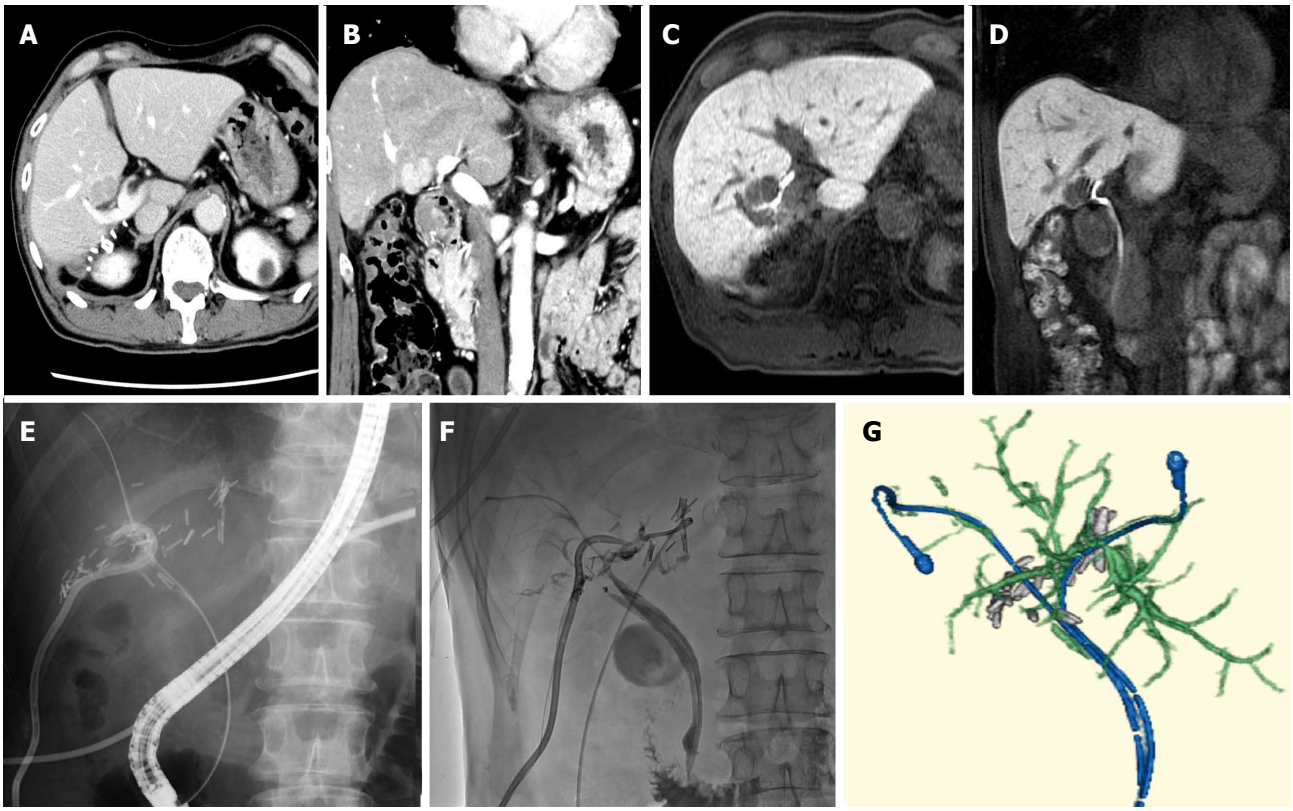

Figure 1 Representative grade C case in bile leakage. A 67-year-old man had hepatocellular carcinoma (diameter: 2 cm; A: Axial view; B: Coronal view) in segment S5 of his liver (located at the bifurcation of the bile duct in the hilar plate) (C: Axial view; D: Coronal view). The tumor was resected via enucleation; E: Bile leakage was detected and so endoscopic retrograde cholangiodrainage was performed together with percutaneous drainage of the resected pouch; F: Subsequently, stenosis of the left hepatic duct due to bile duct ischemia occurred. Percutaneous transhepatic cholangiodrainage was performed via the B3 duct; G: Three-dimensional reconstruction based on CT images obtained before the patient was discharged from hospital. CT: Computed tomography.

Table 1 Comparison between the modified grading system and the Clavien-Dindo classification

Modified grades	Clavien-Dindo classification	
Grade A	Grade Ⅰ	Any deviation from the normal postoperative course that did not require special treatment
	Grade Ⅱ	Cases requiring pharmacological treatment
Grade B	Grade Ⅲa	Cases requiring surgical or radiological interventions without general anesthesia
Grade C	Grade Ⅲb	Cases requiring surgical or radiological interventions performed under general anesthesia
	Grade Ⅳa	Life-threatening complications involving single organ dysfunction
Grade D	Grade Ⅳb	Life-threatening complications involving multiple organ dysfunction
	Grade Ⅴ	Cases that resulted in death

Table 2 Grading system and representative management strategies for post-hepatectomy liver failure

Grades	Definition	Management strategies
Grade A	No change in the patient's clinical management strategy required or manageable with medication	Diuretics, selective digestive decontamination, lactulose, glucagon-insulin therapy, stronger neo-minophagen C
Grade B	Manageable without invasive treatment	FFP transfusion, hyperbaric oxygen therapy
Grade C	Invasive treatment required	Plasma exchange, artificial liver support, surgery (including liver transplantation)

Artificial liver support is including high-flow hemodialysis with FFP transfusion. FFP: Fresh frozen plasma.

ASCITES

Ascites is a common complication in patients who exhibit liver dysfunction or cirrhosis after liver resection[27]. One of the possible pathogenic mechanisms of the ascites seen after liver resection is portal flow resistance at the sinusoidal level due to a reduction in the volume of the portal vascular bed[28]. Hepatic outflow block can also cause increased portal flow resistance[29]. The acute phase after liver resection tends to involve edema in the interstitial organ space, which leads to increased portal flow resistance. The management of ascites after liver resection

Table 3 Grading system and representative management strategies for bile leakage

Grades	Definition	Management strategies
Grade A	No change in the patient's clinical management strategy required or manageable with simple drainage	Drainage within 7 d Antibiotic administration
Grade B	Manageable with interventional procedures	Drainage for 7 or more day, ethanol injection, fibrin paste injection, single ENBD, single EBD, single PTBD, PTPE, TAE
Grade C	Cases involving pneumoperitoneum, inflammation, multiple organ failure, or reoperation	Complicated IVR (combinations with any Grade Bs) Reoperation

ENBD: Endoscopic nasobiliary drainage; EBD: Andoscopic biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; PTPE: Percutaneous transcatheter portal embolization; TAE: Transcatheter arterial embolization; IVR: Interventional radiology.

Table 4 Grading system and representative management strategies for acute renal failure

Grades	Definition	Management strategies
Grade A	Increase in serum creatinine level of ⩾ 0.3 mg/dL from the baseline or 1.5 to 2-fold increase from the baseline Urinary output of less than 0.5 mL/kg per hour for more than 6 h	Dehydration Diuretics
Grade B	Two-fold increase in the serum creatinine level from the baseline Urinary output of less than 0.5 mL/kg per hour for more than 12 h	Continuous mannitol + diuretics
Grade C	Dialysis treatment required (serum K > 6.0 mEq, BE < -10, uremia, hypouresis that lasts for more than three days)	Hemodialysis

Table 5 Grading system and representative management strategies for ascites

Grades	Definition in International Ascites Club (2003)	Definition in International Ascites Club (1996)
Grade A	Detected only on United States	Mild
Grade B	Moderate symmetrical distention of the abdomen	Moderate
Grade C	Marked abdominal distention	Massive or tense

Table 6 Grading system and representative management strategies for ascites

Grades	Definition	Management strategies
Grade A	Requiring any changes in the clinical management strategy or manageable with medication Ascites discharge < 1000 mL/d in the drainage case	Diuretics, sodium restriction
Grade B	Grade A ascites that lasts for more than 2 wk or requires peritoneal puncture Ascites discharge < 2000 mL/d in the drainage case	Peritoneal puncture
Grade C	Invasive treatment required	Denver peritoneovenous shunt, TIPS, PSE, splenectomy

TIPS: Transjugular intrahepatic portosystemic shunt; PSE: Partial splenic embolization.

focuses on decreasing the patient's portal pressure[27,28]. The use of diuretics or sodium restriction can decrease systemic flow volume, and ascites can also be controlled by decreasing edema in the inter-organ space or establishing a systemic shunt. Invasive management aims to decrease the patient's portal pressure through mechanical interventions. The IASC previously released statements containing revised definitions of ascites (Table 5); however, they were too abstract to use in academic studies. So, we proposed a modified grading system for post-operative ascites after liver resection (Table 6).

SURGICAL SITE INFECTIONS (SUPERFICIAL, ORGAN AND DEEP) AND WOUND DEHISCENCE

Surgical site infections (SSI) are common after all types of surgery and are classified into superficial, deep incisional, and organ/space SSI. Although several classifications of SSI have been proposed[30], the definitions developed by the Centers for Disease Control and Prevention (CDC) are widely used internationally[31]. According to the CDC, SSI are infections that occur within 30 d of surgery or within one year if an implant is present[31]. In addition, one of the following criteria must be met: (1) purulent drainage from an incision (incisional infection) or from a drain below the fascia (deep infection); (2) a surgeon or attending physician diagnosing an SSI; (3) an infective organism being isolated from a culture of fluid or tissue obtained from the surgical wound (for incisional infections); (4) spontaneous dehiscence or a surgeon deliberately re-opening the wound in the presence of fever or local pain, unless subsequent cultures were negative, or an abscess being detected during direct examinations (for deep infections). However, the grading of SSI based

Table 7 Grading system for superficial SSI and wound dehiscence

Grades	Definitions	Management strategies
Grade A	Manageable within 2 wk	Small open wound, outpatient service
Grade B	Requiring any management 2 wk and more	Large open wound, inpatient service
Grade C	Any management required under general anesthesia	

Table 8 Grading system for deep and organ/space surgical site infections

Grades	Definitions	Management strategies
Grade A	Manageable without requiring any additional perioperative management within 2 wk	Antibiotics, simple drainage
Grade B	Requiring any management 2 wk and more	Additional drainage, irrigation
Grade C	Any management required under general anesthesia	

Table 9 Grading system and representative management strategies for coagulation disorders

Grades	Definition	Managements
Grade A	Does not require any change in the clinical management strategy Plat < 10×10^4 (preoperative Plat was within normal range) 30% reduction in Plat (preoperative Plat was abnormal)	Vitamin K, ATIII, LMWH, SPI, UFH, and DS
Grade B	Medication required for more than 5 d Plat < 5×10^4 (preoperative Plat was within normal range) 60% reduction in Plat (preoperative Plat was abnormal)	Platelet transfusion
Grade C	Intensive care treatment required and involved the failure of other organs	

Plat: Platelet count; ATIII: Anti-thrombin; LMWH: Low molecular weight heparin; SPI: Synthetic protease inhibitor; UFH: Unfractionated heparin; DS: Danaparoid sodium.

Table 10 Grading system and representative management strategies for pneumonia and respiratory disorder

Grades	Definition	Managements
Grade A	Meet SIRS criteria with imaging findings in less than 50% of the lung field or PaO_2/FiO_2 < 300	Antibiotics and oxygen Sputum suction
Grade B	Meet SIRS criteria with imaging findings in 50% and more of the lung field or PaO_2/FiO_2 < 200	Antibiotics and oxygen, IPPV, NPPV, bronchoscopy for sputum suction
Grade C	Requiring ventilator support	Ventilator

Systemic inflammatory response syndrome criteria is defined as two or more of the following clinical signs: bodily temperature > 38 ℃ or < 36 ℃, heart rate > 90/min, respiratory rate > 20 /min or $PaCO_2$ < 32 mmHg, WBC > 12000/μL or < 4000 /μL or immature cells > 10%. Pneumonia imaging is any of air-space opacity, lobar consolidation, or interstitial opacities. SIRS: Systemic inflammatory response syndrome; IPPV: Intermittent positive-pressure breathing; NPPV: Nasal positive-pressure ventilation.

on symptoms and the management strategy employed is difficult. Therefore, we proposed that SSI should be graded based on how long they take to cure (Table 7 for superficial SSI and wound dehiscence, Table 8 for deep and organ/space SSI). Using this new grading system, it is very easy and simple to grade SSI objectively.

COAGULATION DISORDERS

Coagulation disorders are a common complication after liver resection[32,33]. Most coagulation and anti-coagulant factors are synthesized by the liver, and the ability to synthesize such factors rapidly deteriorates after liver resection in cirrhotic patients and those who experience marked hepatic volume loss[20]. In addition, most patients who are scheduled to undergo liver resection present with thrombocytopenia due to portal hypertension. Therefore, a prolonged prothrombin time, a prolonged thrombin time, elevated levels of fibrinogen degradation products, and a low platelet count are common after liver resection[34]. As we have mentioned in the ascites section, portal hypertension can occur after liver resection due to an increase in portal flow resistance[17]. Therefore, coagulation disorders should be divided into two different grades based on whether the patient displays normal or abnormal preoperative platelet levels (Table 9).

PNEUMONIA AND RESPIRATORY DISORDER

Postoperative pneumonia and respiratory disorder (PPN/RD) was rarely seen after liver resection recently except in the elderly cases[35,36]. Definition of the PPN/RD

was shown in Table 10. Clinical sign of the PPN/RD is systemic inflammatory response syndrome with any radiological imaging findings[37]. Management will be taken by administrating susceptible anti-biotics with oxygen supply. Acute lung injury (ALI) is defined by PaO_2/FiO_2 ratios < 300 and acute respiratory distress syndrome (ARDS) is defined by PaO_2/FiO_2 ratios < 200[38]. In our grading, ALI is in Grade A and ARDS is in the grade B (Table 10). Our grading is not only defined PPN/RD after liver resection but also after other general surgery.

CONCLUSION

The complications seen after liver resection are different from those encountered after other types of surgery because the liver produces most serum proteins, which play a major role in maintaining systemic homeostasis, and liver resection affects liver function. Therefore, post-liver resection complications tend to be severe. The risk factors for complications after liver resection depend on the pathological background of the liver itself[39]. In patients with normal liver function, the operative time, fresh frozen plasma transfusion requirement, tumor size, and retinol binding protein levels are independent risk factors for complications[40]. On the other hand, the PT and the indocyanine green retention value at 15 min are independent risk factors for complications in cirrhotic patients[40]. Therefore, consensus definitions and grading systems are necessary to allow comparisons between academic reports. Our grading system incorporates established consensus definitions and statements, such as those for PHLF and BL, and attempts to establish objective definitions for grading other complications. We hope that our grading system will be used to describe the complications experienced after liver resection.

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RETROSPECTIVE STUDY

Role of autophagy in differential sensitivity of hepatocarcinoma cells to sorafenib

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Abstract

AIM: To investigate the role of sorafenib (SFN) in autophagy of hepatocellular carcinoma (HCC). We evaluated how SFN affects autophagy signaling pathway in human HCC cell lines.

METHODS: Two different human HCC cell lines, Hep3B and Huh7, were subjected to different concentrations of SFN. Cell viability and onset of apoptosis were determined with colorimetric assay and immunoblotting analysis, respectively. The changes in autophagy-related proteins, including LC3, ULK1, AMPK, and LKB, were determined with immunoblotting analysis in the presence or absence of SFN. To assess autophagic dynamics, autophagic flux was measured with chloroquine, a lysosomal inhibitor. The autophagic responsiveness between different HCC cell lines was compared under the autophagy enhancing conditions.

RESULTS: Hep3B cells were significantly more resistant to SFN than Huh7 cells. Immunoblotting analysis revealed a marked increase in SFN-mediated autophagy flux in Huh7 cells, which was, however, absent in Hep3B cells. While both starvation and rapamycin enhanced autophagy in Huh7 cells, only rapamycin increased autophagy in Hep3B cells. Immunoblotting analysis of autophagy initiation proteins showed that SFN substantially increased phosphorylation of AMPK and consequently autophagy in Huh7, but not in Hep3B cells.

CONCLUSION: The autophagic responsiveness to SFN is distinct between Hep3B and Huh7 cells. Resistance of Hep3B cells to SFN may be associated with altered autophagy signaling pathways.

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Key words: Autophagy; Liver cancer; Sorafenib

Core tip: Hepatocellular carcinoma (HCC) is difficult to treat. Sorafenib (SFN) is one treatment option. Autophagy has been proposed to play a pivotal role in HCC. In the present study we investigated the role of autophagy in SFN-treated HCC cells. We found that the autophagic responsiveness to SFN is markedly distinct between Hep3B and Huh7 cells.

Fischer TD, Wang JH, Vlada A, Kim JS, Behrns KE. Role of autophagy in differential sensitivity of hepatocarcinoma cells to sorafenib. *World J Hepatol* 2014; 6(10): 752-758 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i10/752.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i10.752

INTRODUCTION

Hepatocellular carcinoma (HCC), typically occurring in the setting of cirrhosis and chronic hepatitis, is fifth most common cancer diagnosed worldwide and more than 600000 patients are dying of this disease each year[1]. The

incidence of HCC is also rising in the United States. Despite recent advances in the screening and management of HCC, treatment of this pernicious disease is still far from complete, mostly due to its complex mechanisms underlying proliferation, tissue invasion and metastasis of HCC.

Therapies for HCC include chemoembolization, ablation, surgical resection and transplantation[2], but these interventions are highly invasive and often require prolonged hospitalization of the patients. Recently, sorafenib (SFN), an oral multi-kinase inhibitor, has been shown to inhibit tumor-cell proliferation and tumor angiogenesis through its inhibition of vascular endothelial growth factor receptor 2 and other receptor tyrosine kinases[3,4]. In a placebo-controlled phase Ⅲ study, SFN displayed about 3-mo extension of survival in advanced and inoperable HCC cases, leading to Food and Drug Administration (FDA) approval[5]. It is, however, noteworthy that some HCC patients show unresponsiveness or acquired resistance to SFN[6]. It is unclear why the efficacy of SFN is limited, although the activation of survival pathways like PI3K/AKT has been proposed to cause the development of SFN resistance[7].

Autophagy is an evolutionary conserved cellular process that degrades both long-lived cytoplasmic proteins and surplus or dysfunctional organelles by lysosome-dependent machinery[8]. Impaired and insufficient autophagy is causatively linked to pathogenesis of ischemia/reperfusion injury and drug-induced toxicity in the liver[9-11]. Growing evidence is accumulating that autophagy also plays a pivotal role in carcinogenesis, tumor proliferation, and resistance to chemotherapy[12]. In addition, recent studies on an anti-cancerous role of autophagy raise a possibility that the modulation of autophagy could be a new therapy against cancer[13,14]. However, a pro-cancerous role of autophagy has also been suggested[15]. Thus, the precise role of autophagy in HCC is largely yet to be elucidated.

In the present study, we investigated the role of autophagy in HCC using two human HCC cell lines, Hep3B and Huh7 cells. Our results demonstrate that autophagic response to SFN and autophagy signaling pathways are markedly distinct between these two HCC cells.

MATERIALS AND METHODS

Reagents and chemicals

SFN and rapamycin were purchased from LC Laboratories (Woburn, MA) and dissolved in DMSO. Chloroquine was purchased from Sigma Chemical Co (St. Louis, MO) and dissolved in phosphate-buffered saline (PBS; 2.7 mmol/L KCl, 137 mmol/L NaCl 10.1 mmol/L Na_2HPO_4, and 1.8 mmol/L KH_2PO_4, pH7.4). Antibodies against ULK1 were purchased from Sigma Chemical Co (St. Louis, MO). All other primary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Cell culture

The human HCC cell lines, Hep3B and Huh7 cells, were purchased from American Type Culture Collection (Manassass, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassass, VA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Mediatech) in 5% CO_2 at 37 ℃. Cells were used for experiments at approximately 80% confluence. For immunoblotting experiments, cells were plated in 60 mm culture dishes at 6×10^5 cells. DMSO was used for a vehicle control. To induce nutrient depletion and starvation, cells were incubated in Krebs-Ringer-hydroxyethylpiperazine-N-2 ethanesulfonic acid (HEPES) (KRH) medium containing 115 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, and 25 mmol/L HEPES at a pH of 7.4.

MTT viability assay

To determine cell viability, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-biphenyl tetrazolium (MTT) assay was used with the different concentrations of SFN and rapamycin. HCC cells were plated on a 96-well microplate at 5×10^3 cells per well for up to 72 h in DMEM medium. DMSO was used as a vehicle control. The MTT salt dissolved in PBS (5 mg/mL) was added to the medium and incubated for 2 h[16]. The medium was subsequently removed and replaced with isopropyl alcohol. The optical density was read at 562 nm in SpectraMax M2e Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The results were shown as a ratio of viability of treated to vehicle groups.

Immunoblotting for autophagy proteins

Whole cell lysates were prepared by extracting proteins with radioimmunoprecipitation (RIPA) buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 8, 0.1% sodium dodecylsulfate, 1% sodium deoxycholic acid, 1% TritonX-100 and 5 mmol/L EDTA) with 1% protease and 1% phosphatase inhibitors. Protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL). Proteins (10 or 15 μg) were separated by the electrophoresis through 4%-12% polyacrylamide gels (InVitrogen, Carlsbad, CA) or by sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride or nitrocellulose membranes. The expression of LC3, LKB1, phospho-AMPKα (Thr172), AMPKα, PARP and GAPDH were detected using primary polyclonal antibodies. After overnight incubation with primary antibodies at 4 ℃, the membranes were incubated with donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently visualized by enhanced chemiluminescence. Changes in protein expression were determined using the ImageJ software (National Institutes of Health, Bethesda, MD)

SiRNA-mediated knockdown of AMPK

Small interfering RNA (siRNA) for AMPKα1/α2 (sc-45312) and the siRNA Reagent System (sc-45064) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). In a six well tissue culture plate, Huh7 cells were cultured until approximately 70% confluent. For

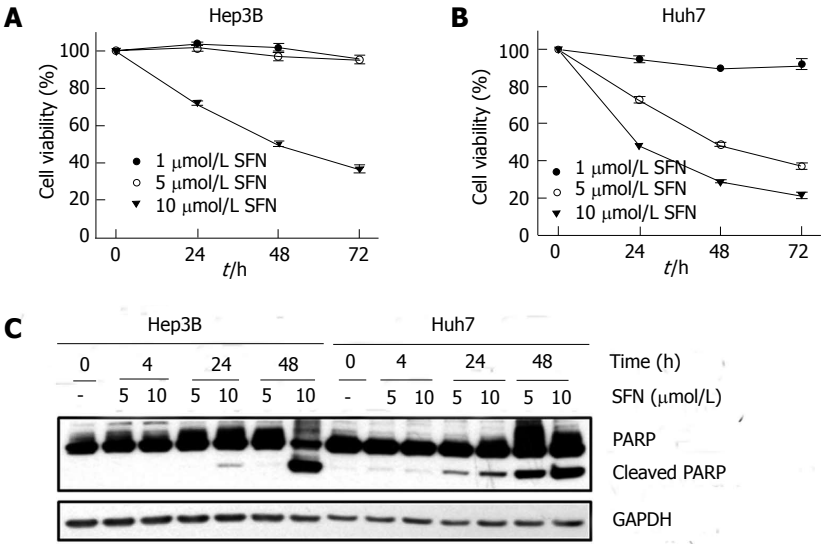

Figure 1 Cell death with sorafenib. The effect of varying doses (1, 5 and 10 μmol/L) of SFN on hepatocellular carcinoma cell viability was determined by the MTT assay at 24, 48 and 72 h in (A) Hep3B and (B) Huh7 cells. PARP expression was analyzed by immunoblotting analysis (C). SFN: Sorafenib; RAPA: Rapamycin.

each transfection, 80 pmol of siRNA was diluted into 100 μL of siRNA transfection medium, as suggested by the manufacturer. Huh7 cells were incubated with siRNA transfection reagents. Scrambled siRNA (sc-37007) was used for the control experiments.

Statistical analysis

Results were evaluated using unpaired two-tailed Student's *t* test. Data are expressed as mean ± SE and $P < 0.05$ denotes statistical significance. All values are representative of at least three different experiments per group.

RESULTS

Chemoresistance of Hep3B to SFN

To determine the chemosensitivity of HCC cells to SFN, Hep3B and Huh7 cells were treated with different concentrations of SFN for up to 72 h and cell death was evaluated with the MTT assay (Figure 1). There was a marked difference of cell death between two cells. While 5 μmol/L SFN induced virtually no cell death in Hep3B, this concentration caused a significant cell death in Huh7 in a time dependent manner (Figure 1A). Although 10 μmol/L SFN induced cell death in both cell types, the extent of cell killing was substantially greater in Huh7 cells than in Hep3B cells. To confirm the differential sensitivity to SFN between two cell types, the onset of apoptosis was determined with immunoblotting of poly (ADP ribose) polymerase (PARP) cleavage (Figure 1B). Similar to the results from the MTT assay, SFN substantially induced apoptotic cell death in Huh7 cells. Taken together, these results suggest that Hep3B cells are intrinsically more resistant to SFN than Huh7 cells.

Different autophagic responsiveness between Hep3B and Huh7 cells

Autophagy is a pro-survival mechanism in normal cells and has also been associated with chemoresistance in cancer cells[17-19]. Accordingly, we investigated if the autophagic responsiveness to SFN is different between two cells. Microtubule-associated protein 1 light chain 3-Ⅱ (LC3-Ⅱ), a mammalian orthologue of Atg8, is a specific autophagy marker[20]. Immunoblotting of LC3 showed that SFN at three different concentrations barely changed the expression of LC3-Ⅱ in Hep3B cells (Figure 2A). However, in Huh7 cells, 10 μmol/L SFN significantly increased LC3-Ⅱ expression, suggesting that this concentration of SFN alters autophagy in Huh7 cells, but not in Hep3B. Autophagy is a dynamic process between autophagosomal entrapment and autolysosomal clearance[8]. Thus, the increase in LC3-Ⅱ by SFN in Huh7 cells could be due to either an increase in autophagosome formation or a decrease in autolysosome formation. To distinguish these two possibilities, we measured autophagic flux with chloroquine (CQ), a lysosomotropic agent that inhibits autolysosomal clearance[10]. Immunoblotting analysis of LC3 in the presence and absence of CQ revealed that SFN substantially increased autophagic flux only in Huh7 cells (Figure 2B). Therefore, these data demonstrate that Huh7 cells have higher autophagic responsiveness to SFN than Hep3B cells.

Starvation or nutrient depletion is a powerful stimulus for autophagy[9]. Next, we examined autophagic response of two cells to starvation. To induce starvation, cells were incubated in amino acid- and serum-free KRH for up to 4 h and changes in LC3 were evaluated with immunoblotting (Figure 2C). Autophagy was rapidly increased in Huh7 cells during 1 h of starvation, as judged by increased LC3-Ⅱ expression. However, this starvation-induced increase in LC3-Ⅱ was not evident in Hep3B cells, implying that starvation fails to induce autophagy in Hep3B cells.

Rapamycin enhances autophagy through mTOR inhibition[21]. To investigate if rapamycin can induce autophagy in HCC cells, either Hep3B or Huh7 cells were treated with 5 nmol/L rapamycin and changes in LC3 were determined in the presence and absence of CQ (Figure 2D). In a striking contrast to both SFN and starvation, rapamycin increased the expression of LC3-Ⅱ and autophagic flux in Hep3B cells as well as Huh7 cells.

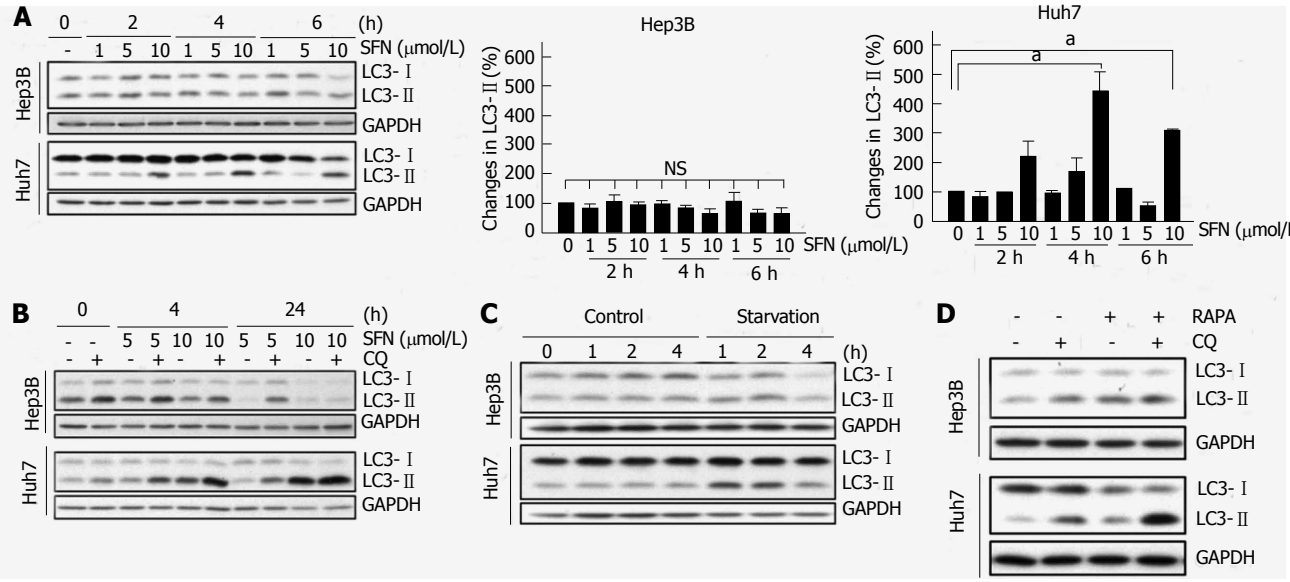

Figure 2 Autophagic response to sorafenib. A: LC3 expression in hepatocellular carcinoma cells was analyzed in the presence of SFN for up to 6 h. Representative blot (left panel) and quantitative analysis of LC3-Ⅱ by densitometry (middle and right panel). [a]$P < 0.05$; B: LC3 expression was analyzed in the presence of SFN with and without CQ to determine autophagy flux after 4 and 24 h; C: Autophagic response to nutrient-rich (control) or nutrient-depleted medium was analyzed by immunoblotting of LC3 in Hep3B and Huh7 cells for up to 4 h; D: LC3 expression was analyzed in the presence of 5 nmol/L RAPA after 4 h treatment with and without CQ to examine autophagy flux. NS: Non-significant; SFN: Sorafenib; CQ: Chloroquine; RAPA: Rapamycin.

These findings suggest that mTOR-mediated autophagy is functional and operative in both Hep3B and Huh7 cells. Collectively, our data demonstrate that the autophagic responsiveness of Hep3B cells to SFN and starvation is markedly distinct from that of Huh7 cells.

Different autophagy signaling between Hep3B and Huh7 cells

Autophagy is a multi-step process consisting of initiation, elongation and completion[8]. At the initiation stage of autophagy, two proteins play an integral role in cargo selection and phagophore formation: 5'-adenosine monophosphate-activated protein kinase (AMPK), a molecular energy sensor, and Unc-51 Like Autophagy Activating Kinase 1 (ULK1)[22], a mammalian homolog of yeast Atg1. To investigate whether SFN affects these proteins, we analyzed changes in AMPK and ULK1 expression with immunoblots (Figure 3). Densitometric analysis revealed a significant difference in the status of phospho-AMPK (p-AMPK) between two cells when SFN was added (Figure 3A and B). While the basal levels of p-AMPK were comparable between two cells, administration of SFN to Huh7 cells significantly increased p-AMPK expression. Changes in p-AMPK in Hep3B in the presence of SFN were, however, minimal. Total AMPK levels remained unchanged in both cells. Interestingly, the basal levels of liver kinase B1 (LKB1), a Ser/Thr kinase phosphorylating AMPK[23], was also significantly higher in Huh7 cells than in Hep3B cells (Figure 3A and C). When Huh7 cells were treated with SFN, the expression of LKB1 gradually decreased but remained higher than Hep3B cells especially during the early period of SFN treatment. On the contrary, the basal levels of ULK1 were significantly higher in Hep3B cells than in Huh7 cells (Figure 3A and D). Administration of SFN did not change ULK1 expression in both cell types throughout 24 h of treatment. Therefore, these results demonstrate that the initiation signaling pathways of autophagy are noticeably different between two HCC cells.

The role of AMPK in autophagy of HCC cells

To further investigate how AMPK affects autophagy in Huh7 cells, AMPK was silenced with siRNA-mediated approaches (Figure 4A). Knocking down of AMPK substantially increased the levels of LC3-Ⅱ, suggesting an integral role of AMPK in the basal autophagy of Huh7 cells. Co-addition of SFN further enhanced LC3-Ⅱ expression more than 300%, as compared to the treatment with siRNA alone, implying that SFN may regulate multiple targets of autophagy process other than AMPK. Notably, CQ did not increase LC3-Ⅱ under this condition.

Next, we explored the effects of AMPK activation on HCC cell death. HCC cells were treated either with 5 mmol/L metformin, an AMPK activator, or with 10 μmol/L SFN for 24 h. Some cells were treated with both. In Hep3B cells, metformin itself significantly increased cell death, which was further enhanced by SFN (Figure 4B). On the contrary, the addition of metformin failed to increase SFN-dependent cell death in Huh7 cells, suggesting that the responsiveness to metformin in the presence of SFN is distinct between two cells.

DISCUSSION

HCC is prevailing worldwide and more than 20000 new cases are reported in the United States each year[1]. The onset of HCC from hepatitis C virus infection is the one of the fastest-rising cause of cancer-mediated death in

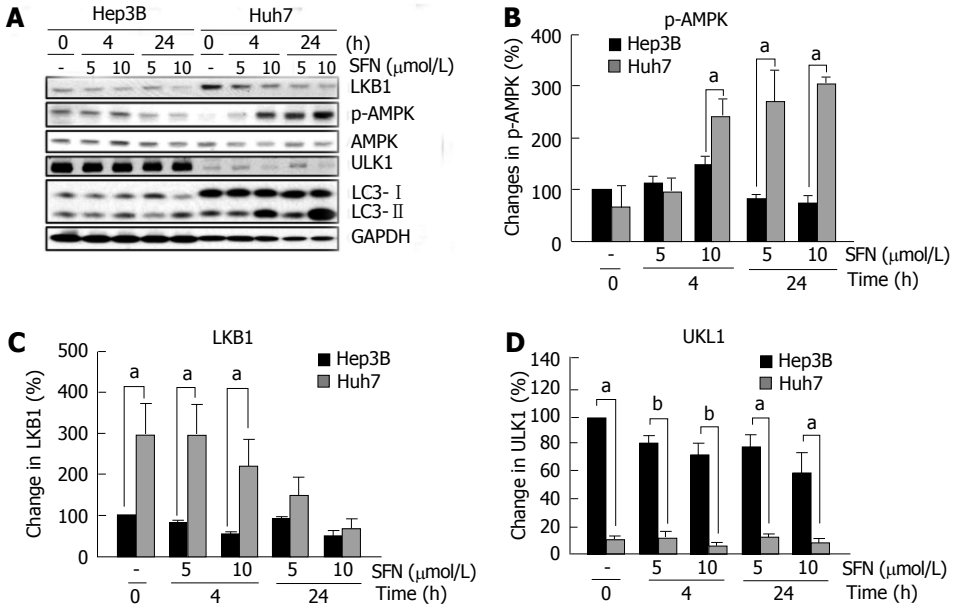

Figure 3 Altered autophagy signaling in hepatocellular carcinoma cells. A: Hepatocellular carcinoma cells were treated with 5 and 10 μmol/L SFN for 4 and 24 h and the expression of autophagy initiating proteins was analyzed with immunoblotting; B: Quantitative analysis of p-AMPK. $^{a}P < 0.05$; C: Quantitative analysis of LKB1. $^{a}P < 0.05$; D: Quantitative analysis of ULK1. $^{b}P < 0.001$. SFN: Sorafenib.

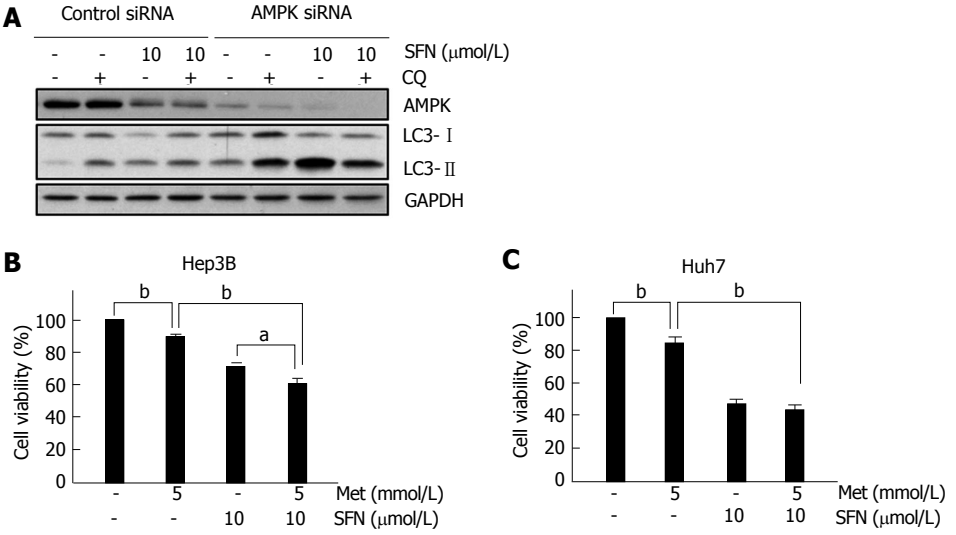

Figure 4 Gene silencing for AMPK. A: Huh7 cells were treated with siRNA for AMPK for 24 h. After 4 h treatment with SFN, LC3 was assessed with immunoblotting assay; B, C: The effect of either Met or SFN on hepatocellular carcinoma cell viability was determined by the MTT assay at 24 h in (B) Hep3B and (C) Huh7 cells. Some cells were treated with both agents. $^{a}P < 0.05$, $^{b}P < 0.001$. Met: Metformin; SFN: Sorafenib; CQ: Chloroquine.

the United States. During the past two decades, the incidence of HCC in the United States has tripled while the survival for 5 years has remained below 12%[24]. Thus, HCC remains a difficult and challenging cancer to treat. SFN is an oral multi-kinase inhibitor and inhibits tumor-cell proliferation and tumor angiogenesis[3,4]. Although this FDA-approved dug can prolong the survival of advanced and inoperable HCC patients, the average extension of survival is limited to about 3-mo. Furthermore, some patients do not respond to SFN[6]. The mechanisms behind such a limited efficacy of SFN remain unknown. Autophagy is an evolutionary conserved cellular process and has been proposed to play a pivotal role in HCC. In the present study, using human HCC cell lines, we show that Hep3B cells are resistant to SFN-mediated apoptosis, while Huh7 cells are prone to apoptosis in the presence of SFN. Furthermore, we demonstrate that SFN induces a substantial enhancement of autophagy in Huh7 cells, but not in Hep3B cells, and that autophagy signaling pathways are noticeably distinct between two HCC cells.

Autophagy mainly plays a pro-survival role in normal cells by providing cells and tissues with nutrients. However, autophagy can cause cell death through a selective degradation of essential proteins and constituents in the cells[25]. The SFN-sensitive HCC cell line, Huh7 cells, exhibited a substantial difference in both cell death and au-

tophagic responsiveness, compared to the SFN-resistance cell line, Hep3B cells (Figures 1 and 2). Immunoblotting analysis of LC3 and autophagic flux showed that SFN induced a marked increase in autophagy in Huh7 cells, which was, however, absent in Hep3B cells (Figure 2). Lack of the autophagic responsiveness in Hep3B cells was also observed under the condition of starvation, a powerful stimulus of autophagy[9,12], suggesting that these two HCC cells have an intrinsically distinct autophagy. Furthermore, our results suggest that the enhancement of autophagy by SFN in Huh7 cells may be associated with the activation of p-AMPK, a key protein involved in autophagy initiation[22] (Figure 3). The importance of p-AMPK in SFN-dependent activation of autophagy is further supported by our findings that Hep3B cells fail to increase p-AMPK expression upon treatment of SFN. The necessity of AMPK activation for autophagy induction in HCC has been recently reported[26,27].

The contradictory effects of SFN on Hep3B and Huh7 cells have been reported[28] and may be linked to different autophagic responsiveness to this agent. Our results show that starvation or nutrient depletion fails to induce autophagy in Hep3B cells (Figure 2C). The absence of autophagy enhancement by either SFN or starvation could stem from defective or impaired autophagy in Hep3B cells. However, autophagic flux analysis revealed that Hep3B cells, indeed, have a considerable basal and mTOR-dependent autophagic capacity, as judged by the increase in LC3-Ⅱ either with CQ (Figure 2B) or with rapamycin (Figure 2D). These results imply that the signaling pathways of starvation-mediated autophagy may be altered in Hep3B cells. Although the mechanisms underlying SFN-induced autophagy remain to be elucidated, lack of autophagic response to both starvation and SFN in Hep3B cells led us to speculate that SFN-dependent autophagy requires a similar signaling pathway of the starvation-mediated autophagy. Since the only known difference in signaling mechanism between starvation- and rapamycin-induced autophagy exists in the initiation stage of autophagy process[8], we reasoned that events upstream to autophagy signaling pathways might be altered in Hep3B cells. In agreement with this view, we observed that the activation of AMPK, a critical event in autophagy induction under the condition of starvation, was evident only in Huh7 cells, but not in Hep3B cells, upon SFN administration. The precise mechanisms behind SNF-induced autophagy warrant future studies.

When normal, non-tumorigenic cells are subjected to stresses such as ischemia/reperfusion, alcohol and drug, autophagy becomes activated as an adaptive response to these stresses[12]. In contrast, autophagy can prevent and promote tumor development. These seemingly contradictory roles of autophagy in tumor stem from the complexity of tumorgenesis[12]. Prior to tumor establishment, autophagy clears damaged organelles and proteins, leading to preventing neoplastic transformation. However, when tumor develops, the demand for metabolic supplies is progressively increasing. As a consequence, autophagy becomes fully activated to provide the tumor cells with nutrients and amino acids. Thus, autophagy is a "double-edged sword" in cancer where it initially acts as a tumor suppressor, but later acts as a tumor promoter when tumor is established[15].

In conclusion, we have shown that Hep3B cells responds differently to various autophagy stimuli, compared to Huh7 cells. Although the modulation of autophagy could have a new therapeutic potential against cancer, our study demonstrates that caution should be taken before considering autophagy as anticancer regimes in HCC patients.

COMMENTS

Background

The incidence of hepatocellular carcinoma (HCC) is prevailing worldwide but its treatment is still disappointing. Sorafenib (SFN), an oral multi-kinase inhibitor, is one treatment option for HCC patients but its efficacy is limited. Autophagy is a cellular catabolic process that degrades both long-lived cytoplasmic proteins and surplus or dysfunctional organelles by lysosome-dependent machinery. Autophagy also plays a pivotal role in carcinogenesis, tumor proliferation, and resistance to chemotherapy. However, the role of autophagy in HCC remains unclear.

Research frontiers

HCC is fifth most common cancer diagnosed worldwide and the incidence of this pernicious disease is also rising in the United States. However, the treatment of HCC is still far from complete.

Innovations and breakthroughs

In this study authors evaluated the effects of SFN on autophagy in HCC cell lines. Authors found that individual HCC cells respond quite differently to various autophagy stimuli due to distinct autophagy signaling pathways between HCC cells.

Applications

Although the modulation of autophagy could have a new therapeutic potential against cancer, authors demonstrate here that caution should be taken before considering autophagy as anticancer regimes in HCC patients.

Terminology

The most important terms in this article are: HCC, SFN and autophagy.

Peer review

The authors have an idea to find SFN effects in HCC cell lines. To this end, they analyzed the changes of autophagy-related proteins in the cells, and found the different responsiveness to SFN involves autophagy signaling pathway. The paper is interesting.

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META-ANALYSIS

Predictability of IL-28B-polymorphism on protease-inhibitor-based triple-therapy in chronic HCV-genotype-1 patients: A meta-analysis

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Abstract

AIM: To investigate the predictability of interleukin-28B single nucleotide polymorphism rs12979860 with respect to sustained virological response (SVR) in chronically hepatitis C virus (HCV) genotype-1 patients treated with a protease-inhibitor and pegylated interferon-α (Peg-INF-α) based triple-therapy.

METHODS: We searched PubMed, the Cochrane Library and Web of Knowledge for studies regarding the interleukin 28B (IL-28B)-genotype and protease-inhibitor based triple-therapy. Ten studies with 2707 patients were included into this meta-analysis. We used regression methods in order to investigate determinants of SVR.

RESULTS: IL-28B-CC-genotype patients achieved higher SVR rates (odds 5.34, 95%CI: 3.81-7.49) than IL-28B-non-CC-genotype patients (1.88, 95%CI: 1.43-2.48) receiving triple-therapy. The line of therapy (treatment-naïve or -experienced for Peg-INF-α) did not affect the predictive value of IL-28B (P = 0.1). IL-28B-CC-genotype patients treated with protease inhibitor-based triple-therapy consisting of Boceprevir, Simeprevir, Telaprevir or Vaniprevir showed odds of 3.38, 14.66, 7.84 and 2.91, respectively. The odds for CC genotype patients treated with Faldaprevir cannot be quantified, as only a single study with a 100% SVR rate was available.

CONCLUSION: IL-28B-SNP predicts the outcome for chronic HCV genotype-1 patients receiving protease inhibitor-based triple-therapy. The predictive value varies between the different protease inhibitors.

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Key words: Hepatitis C virus; Direct antiviral agents; Interleukin 28B; Sustained virological response; Meta-analysis

Core tip: Hepatitis C is a world health problem and represents a dynamic field of research for new therapeutic options. Recently direct antiviral agents such as protease inhibitors have been developed which, in addition to pegylated interferon-α and Ribavirin, obtain higher sustained virological response (SVR) rates. Of note, costs are higher and side effects are more common. The data regarding the predictive value of Interleukin 28B (IL-28B) are controversial. This meta-analysis was conducted on 2707 patients treated with different protease inhibitors. Its aim was to clarify the predic-

tive value of IL-28B on SVR in protease inhibitor-based triple-therapy, allowing the possibility of personalized treatment.

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INTRODUCTION

Hepatitis C virus (HCV) is a global health Problem. According to the World Health Organization, approximately 150 million people are chronically infected with HCV, and it is estimated that more than 350 thousand are dying each year[1]. HCV is responsible in Europe and North America for 50% of liver cirrhosis and 25% of hepatocellular carcinoma[2-4].

HCV has 7 genotypes (1 to 7) and approximately 100 subtypes[5]. Genotype 1, which is the most common HCV genotype in Western countries, has the worst prognosis and response to antiviral treatment in comparison to other genotypes[6-8].

In the last years the standard therapy (Standard of care, SOC) for HCV consisted of pegylated Interferon-α (Peg-IFN-α) and Ribavirin (RBV)[9]. Recently, several direct antiviral agents (DAA) were developed, such as the protease inhibitors (PI) Boceprevir (BOC), Telaprevir (TVR), Vaniprevir (VNP), Faldaprevir (FLP) and Simeprevir (SMP)[10-13]. In pivotal studies, patients treated with BOC or TVR and Peg-IFN-α/RBV achieved significantly higher sustained virological response (SVR) rates compared to standard therapy[11-14]. These new treatment options bring new hopes for chronically HCV infected patients but they have more side effects and higher costs[10].

Treatment predictors are important tools for the management of therapy in patients with chronic HCV infection. For the current standard treatment with Peg-IFN-α/RBV in patients with chronic HCV infection, HCV genotypes 2 and 3, low baseline viral load, ethnicity, younger age, low γ-GT levels, low γ-GT/ALT level, absence of advanced fibrosis/cirrhosis, and absence of steatosis in the liver have been identified as independent pretreatment predictors of a SVR[15,16].

After initiation of treatment, rapid virological response (RVR, undetectable HCV-RNA at week 4 of therapy) is the best predictor of SVR independent of HCV genotype[16]. Recently, several genome-wide association studies showed that a single nucleotide polymorphism (SNP) within the interleukin 28B (IL-28B) gene is significantly associated with treatment outcome under standard treatment in chronically HCV genotype-1 infected patients[17-19]. IL-28B rs12979860 is the most investigated allele of IL-28B in Europe and North America. The data about the predictive value of IL-28B-genotype in HCV genotype-1 and triple-therapy are inconsistent. In the studies with VNP, IL-28B-genotype had no predictive value for the treatment[13,20]. In the studies by Poordad *et al*[21], Fried *et al*[22], Bronowicki *et al*[23], Sulkowski *et al*[24] and Akuta *et al*[25], IL-28B-CC-genotype had a favorable prognosis. In the study by Flamm *et al*[26] for Boceprevir, genotype IL-28B-TT had a favorable prognosis and by Jacobson *et al*[27] and Pol *et al*[28], IL-28B-genotype had a limited influence on SVR. However, more information about the predictability of IL-28B-genotype would allow physicians to individualize antiviral HCV therapy.

Therefore, we conducted this meta-analysis to investigate the predictive value of IL-28B rs12979860 (CC *vs* CT + TT) allele for SVR in chronically HCV genotype-1 infected patients treated with a triple-therapy regimen consisting of a DAA (BOC, TVR VNP, FLP or SMP) and Peg-IFN-α/RBV.

MATERIALS AND METHODS

We searched in PubMed, Web of Knowledge and the Cochrane Library databases, for relevant articles (full text and meeting abstracts) up to January 2014 regarding the following the next key words: "Boceprevir" or/and "SCH503034", "Telaprevir" or/and "VX-950", "Ciluprevir" or/and "BILN 2061", "Simeprevir" or/and " TMC435", "Danoprevir" or/and "R7227", "Vaniprevir" ("MK-7009"), "MK-5172", "Faldaprevir" ("BI201335"), "Narlaprevir" ("SCH900518"), "Asunaprevir" ("BMS-650032"), "PHX1766", "GS-9256", "GS-9451", "ABT450", "IDX320", "ACH-1625". All these DAAs were used as search words in order to avoid missing studies which have determined IL-28B polymorphism for a triple therapy. Because a large number of patient samples were retrospectively tested for IL-28B genotype and some of these results were only presented in meetings, we have decided to include also the meeting abstracts in our meta-analysis. In order to identify relevant studies, the references of the articles included were manually searched. We did not find any other articles that corresponded to our inclusion criteria. The studies search was performed using manual search for Cochrane Library and EndNote X7 for PubMed and Web of Knowledge databases.

The inclusion criteria were: studies with human subjects, more than 18 years of age, HCV genotype-1 patients, treatment with triple-therapy (IFN therapy-naïve and -experienced) with determined IL-28B genetic polymorphism for rs12979860 allele. Only articles in English were included. The exclusion criteria were: HCV/HIV or HCV/HBV co-infection, liver transplantation recipients, pediatric studies and IL-28B genetic polymorphism other than rs12979860. SVR was defined as undetectable HCV-RNA 24 wk after end of treatment.

The studies were reviewed independently by two authors (NCM and AA). All differences were resolved by consensus among these two authors. Our analysis was based on the original published data. For consistency we refrained from contacting the authors of the individual studies. From the studies, the following data were ex-

Table 1 Characteristics of included trials

Ref.	DAA type	Patient type	IL-28B SNP (*n*)	DAA SVR (*n*)		DAA Non SVR (*n*)		SOC SVR (*n*)		SOC Non SVR (*n*)	
				CC	Non CC	CC	Non CC	CC	Non CC	CC	Non CC
Akuta *et al*[25]	TVR	Mixed	68	31	10	6	21				
Bronowicki *et al*[23]	TVR	Naïve	141	30	30	2	48	7	6	4	14
Flamm *et al*[26]	BOC	Experienced	146	12	52	7	24	5	6	5	35
Fried *et al*[22]	SMP	Naïve	153	34	56	1	16	12	17	0	17
Jacobson *et al*[27]	TVR	Naïve	454	84	127	11	71	35	25	20	81
Lawitz *et al*[20]	VNP	Experienced	131	14	67	9	16	1	3	2	19
Manns *et al*[13]	VNP	Naïve	65	22	14	3	10	4	6	1	5
Pol *et al*[28]	TVR	Experienced	527	60	209	16	137	5	13	12	75
Sulkowski *et al*[24]	FLP	Naïve	110	22	34	0	14	9	12	2	17
Poordad *et al*[21]	BOC	Naïve	653	107	198	25	106	50	43	14	110
		Experienced	259	39	105	11	52	6	10	7	29

DAA: Direct acting agents; TVR: Telaprevir; BOC: Boceprevir; SMP: Simeprevir; VNP: Vaniprevir; FLP: Faldaprevir; CC or non CC: Genotype of IL-28B.

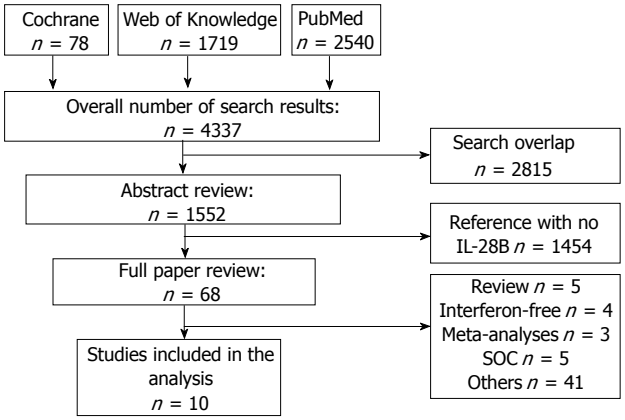

Figure 1 Flow chart of systematic review of protease inhibitor based triple therapy.

tracted: First author, year of publication, type of patients (IFN therapy-naïve or -experienced), total number of patients, the number of patients with determined IL-28B-genotype, type of DAA, IL-28B genetic polymorphism.

The statistical analysis was performed by CR. We used logistic regression to model the chance of a SVR and investigate potential influential factors. In a logistic regression, binary outcome data are modeled based on the *odds* of events (here: SVR). As is usual regression, the *odds* are then formulated as a function of (potential) explanatory variables. Random effects were included in order to accommodate heterogeneity between studies[29]. As the available data allow to fit a multitude of plausible variations of regression models to the data, we approached the *model selection* problem *via* Bayesian Information Criterion (BIC)[30], which allows to compare and select models based on a single adequacy measure. All analyses were performed using the *R* software (www.r-project.org) and the *lme4* package.

RESULTS

Literature search

Four thousand three hundred and thirty-seven studies were initially identified on the bases of DAAs. After removing duplicate citations, the remaining 1522 studies were searched for data regarding IL-28B polymorphism and qualified for abstract review. Among the remaining studies, 1454 studies had no data regarding IL-28B and were excluded. The rest 68 studies were selected for a "full paper review". Among these remaining 68 studies, five of them were reviews. Four of them included only interferon-free therapy. There were three meta-analyses which were excluded. Five studies described only SOC therapy. Another 41 studies and meeting abstracts, including preliminary and subgroup analysis from large trials data, rs8099917 IL-28B allele and non-human studies, had to be excluded (Figure 1).

This meta-analysis is based on the following 10 studies: 7 full text studies and 3 meeting abstracts with a total of 2707 IL-28B patients. The studies of Akuta *et al*[25], Bronowicki *et al*[23], Jacobson *et al*[27] and Pol *et al*[28] investigated the interaction between IL-28B genotype and SVR in patients receiving TVR based triple-therapy. The study from Akuta *et al*[25] had no patients with IL-28B genotype receiving SOC. The studies of Flamm *et al*[26] and Poordad *et al*[21] analyzed the BOC based triple-therapy. VNP was used as DAA in the studies of Lawitz *et al*[20] and Manns *et al*[13]. For SMP and FLP only one study could be included for each of them (Fried *et al*[22] and Sulkowski *et al*[24]; Table 1).

Comparison of dual and triple therapy

Figure 2 illustrates the estimated *odds* and associated *confidence intervals* of a SVR, contrasting dual and triple therapy, and CC and non-CC genotypes. When using conventional dual therapy, the *odds* for SVR are around 0.34 for non-CC genotype (corresponding to Pr = 25% probability), which increases to 1.98 (Pr = 66%) for CC genotype. For triple therapy the *odds* are more favorable, 1.88 (Pr = 65%) for non-CC and 5.34 (Pr = 84%) for CC genotype. The interaction effect between genotype and type of therapy is significant ($P = 0.00126$), *i.e.*, the *odds ratio* between genotypes differs between therapy types (and vice versa). According to the BIC, this model, including a treatment indicator (double *vs* triple), a genotype effect and their interaction fits the data best models that we investigated. In addition including subsets of the

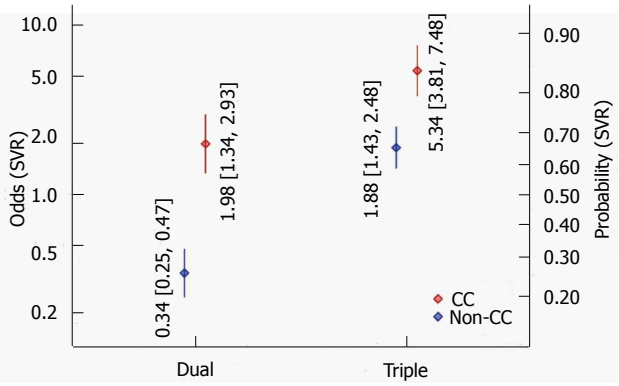

Figure 2 Odds/probabilities of obtaining a sustained virological response with regard to interleukin 28B-genotype and different therapy regimen. The differences between the shown estimates correspond to the odds ratios. A greater difference of odds between the both IL-28B-genotype corresponds to a more beneficial effect. SVR: Sustained virological response; IL-28B: Interleukin 28B.

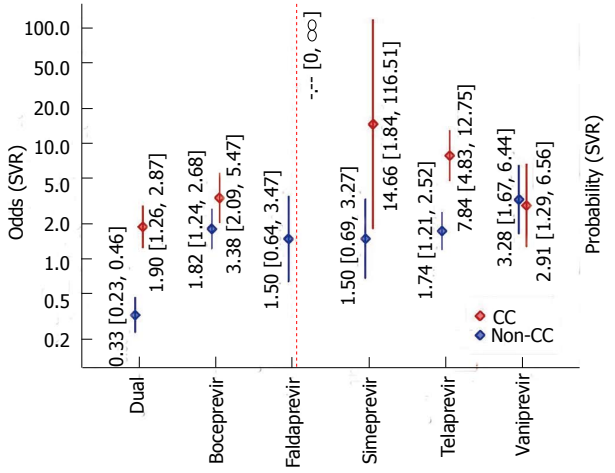

Figure 3 Odds/probabilities of a sustained virological response with regard to interleukin 28B-genotype in different protease inhibitor based triple therapy. The differences between the shown estimates correspond to the odds ratios. A greater difference of odds between the both IL-28B-genotype corresponds to a more beneficial effect. SVR: Sustained virological response; IL-28B: Interleukin 28B.

above variables or use a treatment indicator are also differentiating between different types of DAA.

Comparison of individual DAA types

In addition to the results that came out as best-fitting according to the BIC, we also analyzed the analogous results where protease inhibitor-based triple-therapy is broken down into individual subtypes (DAAs). Comparing this model and the previous one (including interactions in both cases) in an ANOVA, the difference between DAAs actually is significant ($P = 0.0013$). The resulting estimates are illustrated in Figure 3. Among the different DAA types, the estimated odds for SVR tend to be larger than for double therapy and greater for CC than for non-CC genotype. The only two exceptions were VNP, where SVRs for both genotypes appeared to be of the same order of magnitude and FLP, where the CC-*odds* could not be quantified. For FLP, our data originate from a single study with a 100% SVR rate (22 out of 22 patients) for CC genotype; so all we can say is that the evidence is supports effectiveness of FLP in CC genotype patients. Otherwise, for the CC genotype, the greatest odds for SVR are estimated for SMP (OR = 14.66, corresponding to Pr = 94%), whilst for non-CC genotypes, the greatest *odds* are estimated for VNP (OR = 3.28, Pr = 77%). As in the previous model, the interaction effect between treatment type and genotype was significant ($P < 0.001$).

Effect of patient type (IFN-α-treatment-naïve vs IFN-α-experienced)

Consideration of the patient type (IFN-α-treatment-naïve patients *vs* patients having previously experienced IFN-α treatment) in the regression model did not improve the model fit. Even in the best-fitting model among the ones including a patient-type effect, the patient type regarding previously IFN-α therapy was not significant ($P = 0.1$).

DISCUSSION

The main results of this meta-analysis are: (1) IL28B-CC-genotype patients receiving protease inhibitor-based triple-therapy have a higher SVR rate than the IL-28B-non-CC-genotype patients with the same treatment type, (2) considering sub-types of DAAs, the effect appears to be present for BOC, FLP, SMP and TLP, but possibly not for VNP; (3) IL-28B-CC-genotype patients have higher SVR rates both, in IFN-naïve and IFN experienced.

Genome-wide association studies in 2009 showed that different polymorphisms in the region of IL-28B are associated with SVR in patients chronically infected with HCV genotype-1, treated with Peg-INF-α and RBV[17-19]. The IL-28B gene is located on the 19 chromosome. The molecular and immunological mechanism of the IL-28B influence on SVR remains unclear[17-19]. Lately a dinucleotide polymorphism ss469415590 (TT/ΔG) was described to be a better genetic predictor, as IL-28B (INF-λ3) for HCV clearance in chronically HCV genotype-1 infected patients treated with SOC[31-33]. Moreover, only the ΔG of this dinucleotide polymorphism creates a novel type Ⅲ interferon protein, IFN-λ4. Absence of IFN-λ4 protein is thus supposed to favor resolution of HCV infection[31,33].

The determination of IL-28B rs12979860 genotype can help to shorten the therapy duration. Genotyping of IL-28B polymorphisms can further be used to improve patient compliance, to remain on treatment in spite of side effects and to defer treatment in patients with low likelihood of response[34]. The American Association for the Study of Liver Diseases suggests IL-28B polymorphism as a robust predictive marker for treatment decision with Peg-INF-α/RBV or in combination with DAA. Testing is useful if it impacts the treatment decision of either patient or physician. Also in studies with interferon-free therapy regimens IL-28B-CC-polymorphism was associated with better early viral kinetics and higher reduction of viral RNA[35]. Other interferon-free treatment regimens replicated these findings for IL-28B genotypes[36].

Recently, a pangenotypic polymerase inhibitor named sofosbuvir was approved in the United States of America and Europe for the treatment of chronically HCV-infected patients. In selected patients sofosbuvir achieves an SVR rate of approximately 90%. However, a 24-wk therapy with sofosbuvir and ribavirin costs about US$ 169000[37]. Cost-effectiveness analysis show that there is no need to treat patients with IL-28B-CC allelic variation with sofosbuvir urgently because they do not necessarily benefit from such a therapy referring to the SVR rate[38]. Nevertheless, regarding the economic aspects the second-generation protease inhibitors will not be cheaper. For this reason, we need more information about predictive factors in order to detect the individuals who benefit most from an antiviral treatment with polymerase inhibitors. Through the use of predictive factors it will be possible to achieve the highest rate for SVR and the least side effects as well as reducing the cost significantly. Certainly, the IL-28B polymorphisms will play a major role in the future.

Our analyzes showed that IL-28B-CC patients could be treated with a protease inhibitors, either with FLP or SMP. Patients with IL-28B-CC who were treated with either FLP or SMP showed a SVR rate of 100% or 94%, respectively. Therefore, patients with IL-28B-CC genotype could be treated preferably with either FLP or SMP and the IL-28B-non-CC genotypes could be treated preferably with either the polymerase inhibitor sofosbuvir or with a combination of polymerase and protease inhibitors in case of an interferon-intolerance[37].

The difference between the IL-28B SNP predictive effect in triple and dual therapy is significant, suggesting that the effect of IL-28B on the *odds* of a SVR is smaller for triple-therapy than for dual-therapy.

Regarding BOC, the individual studies initially had contradictory results. The study conducted by Flamm *et al*[26] showed that for the IL-28B-TT rs12979860 genotype BOC had a favorable prognosis. However this study had a smaller number of participants than the SPRINT2 and RESPOND2 trials. Poordad *et al*[21] analyzed the data from these studies and showed that IL-28B-CC rs12979860 genotype patients were more likely to achieve a SVR. Our analysis showed that in the case of the patients treated with BOC the CC-genotype has a favorable prognosis.

For FLP and SMP, we could only include one study each, with a relatively small number of participants. For FLP, the *odds* for the CC genotype could not be quantified, due to the fact that our data originate from a single study with 100% SVR rate, indicating a strong beneficial effect.

In the case of SMP, the IL-28B-CC-genotype has the second best *odds* among all DAAs, but with a large *CI* because of the limited number of patients that were included in the study. Therefore, future studies with these DAAs are needed to confirm these results. Our meta-analysis showed that SMP based triple therapy is more likely to produce SVR in CC-genotype patients; therefore we recommend IL-28B genotyping before initiation of this treatment.

The studies by Pol *et al*[28] and Jacobson *et al*[27] showed that IL-28B-genotype has a limited and non-significant predictive value for a SVR regarding the triple-therapy with TVR. Both of them are analyses of the data from larger trials (Pol *et al*[28] from REALIZE and Jacobson *et al*[27] from ADVANCE US) stipulating that TVR based triple-therapy increase the SVR rate through all IL-28B genotypes, especially for the IL-28B-non-CC genotype patients. In our analysis TVR based regimes included a larger number of studies (n = 4). The results were significantly favorable for IL-28B-CC-genotype patients. This result can be explained by the fact that Akuta *et al*[25] studied the predictive value of IL-28B-genotype only in Asian patients infected with genotype 1B, with higher SVR rates while the other studies included wider ranges of ethnicities.

IL-28B SNP has a predictive role for both, IFN-naïve and IFN-previously treated patients. For the SOC-double therapy this meta-analysis did not show evidence for a difference in treatment effect between patient types.

The strong points of our meta-analysis is the large number of patients (n = 2707), the included studies were randomized, controlled studies and the inclusion of various number of DAA types (n = 5). The limitations of our meta-analysis are the relatively small number of studies for some DAAs types (SMP, FLP), even though both, full text and meeting abstracts were included into the search. Another limitation to our study could be the absence of information on the influence of baseline viral loads on SVR and race in correlation with the IL-28B SNP. No long-term data are available yet. Furthermore, this meta-analysis reflects the methodological problems of the included studies.

In conclusion, the IL-28B allelic variation has a predictive value in the protease inhibitor-based triple-therapy of chronically HCV genotype-1 infected patients and it differs among DAA types. However, the effect on the *odds* of a SVR is smaller than the one regarding IL-28B and SOC. We recommend IL-28B genotyping also in the case of SMP-based triple therapy. VNP based regime was the only triple therapy which was not associated with higher SVR rates for IL-28B-CC-genotype patients. Furthermore, prospective studies need to be conducted for the understanding of IL-28B-genotype predictive role in HCV triple-therapy.

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COMMENTS

Background

Hepatitis C is a world health problem and represents a dynamic field of research for new therapeutic options. The interleukin-28B (IL-28B) single nucleotide polymorphism (SNP) is a predictor of sustained virological response for hepatitis C genotype-1 patients treated with pegylated-Interferon-α and ribavirin as the standard of care. Recently, direct antiviral agents have been developed which, in addition to the standard of care, obtain higher sustained virological

response rates, but with higher costs and side effects.

Research frontiers

IL-28B is a solid genetic predictor in the therapy of hepatitis C patients treated with interferon and ribavirin. In the era of new therapeutic options for hepatitis C, the current research hotspot is to evaluate the predictive value of IL-28B in different protease inhibitor-based triple-therapies.

Innovations and breakthroughs

This meta-analysis demonstrates that IL-28B has a predictive value on protease inhibitor-based triple-therapy. This predictability differs among protease inhibitors.

Applications

This study suggests that IL-28B could be used as a genetic predictive factor for antiviral response in hepatitis C genotype 1 patients treated with protease inhibitor-based triple-therapy.

Terminology

Direct antiviral agents such as protease inhibitors are newly developed drugs against hepatitis C. In combination with Interferon and Ribavirin they constitute the triple therapy for hepatitis C. SNP within the interleukin 28B gene as a genetic marker is associated with sustained virological response in the treatment of hepatitis C.

Peer review

Author guidelines has been followed properly in preparing the manuscript. Literature review is adequate. The references are appropriate and relevant. Table and figures reflect the major findings of the study, and they are appropriately presented.

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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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