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EDITORIAL

Long non-coding RNAs era in liver cancer

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies leading to high mortality rates in the general population and the sixth most common cancer worldwide. HCC is characterized by deregulation of multiple genes and signalling pathways. These genetic effects can involve both protein coding genes as well as non-coding RNA genes. Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nt, constituting a subpopulation of ncRNAs. Their biological effects are not well understood compared to small non-coding RNA (microRNAs), but they have been recently recognized to exert a crucial role in the regulation of gene expression and modulation of signalling pathways. Notably, several studies indicated that lncRNAs contribute to the pathogenesis and progression of HCC. Investigating the molecular mechanisms underlying lncRNAs expression opens potential applications in diagnosis and treatment of liver disease. This editorial provides three examples (MALAT-1 metastasis associated lung adenocarcinoma transcript, HULC highly upregulated in liver cancer and HOTAIR HOX transcript antisense intergenic RNA) of well-known lncRNAs upregulated in HCC, whose mechanisms of action are known, and for which therapeutic applications are delineated. Targeting of lncRNAs using several approaches (siRNA-mediated silencing or changing their secondary structure) offers new possibility to treat HCC.

Key words: Hepatocellular carcinoma; Epigenetics; Sequencing; Liver; Long non-coding RNAs

Core tip: The long non-coding RNAs discovery opens a meaningful collision with epigenetics and reveals new roles of RNA in most of cellular processes. This focus explores the functional potentiality of RNAs in the liver in light of most recent knowledge.

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TEXT

Recent advances in massive parallel sequencing, especially RNA sequencing (RNAseq), reveal that at least 90% of the human genome is transcribed into

non-coding RNAs (ncRNAs), while surprisingly less than 2% encodes protein-coding genes. Besides the different types of ncRNAs smaller than 200 nucleotides, such as microRNAs (miRNAs) or PIWI-interacting RNAs, a large proportion of human transcriptome results in RNAs that are longer than 200 nucleotides. Speaking in terms of numbers, this means that about 9000 small ncRNAs and about 32000 long ncRNAs (lncRNAs) have been identified *vs* 21000 protein coding genes[1]. The importance of the lncRNAs has been proven in recent years, as multiple research groups functionally characterized the relevant lncRNAs role in development, epigenetics, cell differentiation and cancer[2]. Basically, lncRNAs can be defined as often polyadenylated RNA, lacking clear open reading frames (ORFs)[2]. The sequence length of this family gives them the ability to have complex secondary structures and to turn inward revealing a tertiary structure[3]. *De novo* discovery and expression analysis of lncRNAs by RNAseq allowed them to be classified along the cell lines, highlighting that lncRNAs expression is strikingly tissue-specific compared with coding genes. Batista *et al*[4] also underline the "address code", both spatial and temporal, of the lncRNAs as key components in cell fate during the development. The repertoire of the functions of lncRNAs seems to be getting more and more increasing and spans between transcription and regulation of messenger RNA (mRNA) processing or translation. They can act *in cis* or *in trans* and the cells can use them to modulate gene expression as well as to bind miRNAs, thereby behaving like a sponge in order to protect the mRNAs target from degradation (ceRNAs)[1].

This editorial focuses on three well-known lncRNAs in the liver and on their potential application as therapeutic targets: metastasis associated lung adenocarcinoma transcript 1 (MALAT-1), highly upregulated in liver cancer (HULC) and HOX transcript antisense intergenic RNA (HOTAIR).

LncRNA MALAT-1 is frequently upregulated in both liver cancer cell lines and hepatocellular carcinoma (HCC) tissue samples; moreover analysis of clinical data demonstrated that its level is an independent prognostic factor for HCC recurrence after liver transplantation[5], potentially acting as a novel biomarker. MALAT-1 is involved in mRNA splicing[6] and may play an essential role in cell cycle regulation[7]. Recent and encouraging studies indicate that antisense oligonucleotides specific to match MALAT-1 disrupt its function, attenuating the corresponding phenotype in cancer cell[8]. A treatment targeting MALAT-1 may be a significant approach in patients following liver transplantation.

One example of ceRNAs class, which has been well characterized in the liver, is HULC. The lncRNA HULC is upregulated in HCC and was found to contain mir-372 binding sites. HULC overexpression can reduce mir-372 level, leading to an induction of PRKACB, which in turn induces CREB phosphorylation[9]. Phosphorylated CREB protein binds to a cAMP response element region, and is then bound to by CBP, which coactivates it, leading to the acetylation of the histone tail and maintaining the open configuration of the chromatin. This regulatory circuitry provides an example of gene reprogramming during tumorigenesis. Interestingly, a recent paper showed that hepatitis B virus X protein (HBx) positively correlated with HULC in clinical HCC tissues. Moreover, HBx also activated the HULC promoter in HepG2 cell lines[10]. Liu *et al*[11] demonstrated that a single nucleotide polymorphism at HULC was associated with decreased sponge activity and decreased HCC risk. It suggests that therapeutic agents that compete with miRNA binding may be useful to treat HCC patients[11].

The third well-known lncRNA is HOTAIR, which is always overexpressed in HCC and liver cancer cell lines. HOTAIR increases PCR2 recruitment to the genomic loci and in this way, it mediates the epigenetic repression of PCR2 target genes, modifying the profile of positive (H3K4me3) or negative (H3K27me3) chromatin marks[12]. Notably, this kind of lncRNA fits into the universe of the chromatin world by changing its structure. An increasing number of chromatin-associated proteins have been implicated in RNA binding, supporting the idea that epigenetic effects are RNA-dependent. Altering the secondary structure of HOTAIR may prevent to embed PCR2 and the consequent aberrant epigenome[13].

All together, these evidences suggest that lncRNAs are strongly associated with liver cancer and they have real potential roles as biomarkers for disease diagnosis, prognosis, or therapeutic response as well as direct targets for therapeutic intervention.

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EDITORIAL

Role of systemic inflammation in cirrhosis: From pathogenesis to prognosis

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Abstract

The natural history of cirrhosis can be divided into an initial stage, known as compensated cirrhosis, and an advanced stage which encompasses both decompensated cirrhosis and acute-on-chronic liver failure (ACLF). The latter syndrome has been recently described as an acute deterioration of liver function in patients with cirrhosis, which is usually triggered by a precipitating event and results in the failure of one or more organs and high short-term mortality rates. Each stage is characterized by distinctive clinical manifestations and prognoses. One of the key elements involved in cirrhosis physiopathology is systemic inflammation, recently described as one of the components in the cirrhosis-associated immune dysfunction syndrome. This syndrome refers to the combination of immune deficiency and exacerbated inflammation that coexist during the course of cirrhosis and relates to the appearance of clinical complications. Since systemic inflammation is often difficult to assess in cirrhosis patients, new objective, reproducible and readily-available markers are needed in order to optimize prognosis and lengthen survival. Thus, surrogate serum markers and clinical parameters of systemic inflammation have been sought to improve disease follow-up and management, especially in decompensated cirrhosis and ACLF. Leukocyte counts (evaluated as total leukocytes, total eosinophils or neutrophil:lymphocyte ratio) and plasma levels of procalcitonin or C-reactive protein have been proposed as prognostic markers, each with advantages and shortcomings. Research and prospective randomized studies that validate these and other markers are clearly warranted.

Key words: Immune dysfunction; Cirrhosis; Acute-on-chronic liver failure; Prognosis; Systemic inflammation

Core tip: Due to the overwhelming evidence that sustains systemic inflammation influences the natural

history of cirrhosis, a review of its current prognostic markers is necessary to highlight their strengths and weaknesses and stimulate further clinical research on this subject.

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INTRODUCTION

Liver cirrhosis is the final phase of all progressive and chronic liver diseases. The natural history of cirrhosis occurs in stages: an initial stage termed compensated cirrhosis and an advanced stage that includes both decompensated cirrhosis and acute-on-chronic liver failure (ACLF), each aspect with distinct clinical manifestations and prognoses[1-4]. The physiopathology of cirrhosis is determined by multiple factors of varying importance, including oxidative stress, systemic inflammation, and organ dysfunction[5]. Systemic inflammation has traditionally been evaluated by the presence of the systemic inflammatory response syndrome (SIRS), a state in which clinical and biochemical parameters such as heart and respiratory rate, white cell count, and body temperature are altered. SIRS is associated with organ dysfunction in cirrhosis patients and with the outcome of ACLF[3,6]. Cirrhosis patients often exhibit systemic inflammation together with immune deficiency as part of the cirrhosis-associated immune dysfunction (CAID) syndrome[6]. Because systemic inflammation contributes to the evolution of cirrhosis, several serum markers and clinical parameters of inflammation have been evaluated as prognostic markers for the late stages of cirrhosis. In this article we outline the stages and physiopathology of cirrhosis, focusing on systemic inflammation, currently-described clinical and biochemical inflammation markers, and their potential utility as prognostic tools.

NATURAL HISTORY OF CIRRHOSIS: THE SLOW LANE AND THE SHORTCUT

If the natural history of cirrhosis is considered from a clinical point of view, the disease can be divided into sequential stages[7] of varying speeds (Figure 1). The traditional clinical classification defines an initial stage, termed compensated cirrhosis, characterized by the absence of complications such as variceal bleeding, ascites, and hepatic encephalopathy. Portal hypertension may already be present (evident by the presence of varices), though below the clinically-relevant threshold[2,8-10]. The initial stage has a low risk for decompensation (7%-10%) and death (1%-3.4%) which is associated with a lower hepatic venous pressure gradient (HPVG)[2,11]. The advanced stage of cirrhosis can be divided according to speed and severity. The "slow lane", termed decompensated cirrhosis, is represented by the multi-step occurrence of cirrhosis-related complications[12]. Progression to decompensated cirrhosis occurs in 5%-7% of compensated cirrhosis patients per year[2]. Sub-classifications of this stage have been suggested by D'Amico *et al*[2], separating patients with ascites with or without esophageal varices that have never bled (associated mortality rate of 20% per year) from those who suffered gastrointestinal bleeding with or without ascites (associated mortality rate of 57% per year). The identified prognostic factors are not only associated with portal hypertension but also with liver function deterioration; thus, these factors include the Child Pugh score, the Model for End-Stage Liver Disease (MELD) score, patient age, and the HPVG. Despite the fact that hemodynamic and clinical variables are key determinants in cirrhosis-associated mortality[7], other events have been linked to poor prognoses. This is the case with bacterial infections, which increase the mortality rate four-fold independently of cirrhosis severity[13-15].

The "shortcut" in the advanced stage is represented by ACLF. This syndrome is defined as an acute deterioration of liver function in patients with cirrhosis, which is usually triggered by a precipitating event and results in the failure of one or more organs and high short-term mortality rates (up to 78% in a three-month period)[3,4,16,17]. ACLF does not always appear as a late or terminal event in cirrhosis, since it can occur in the absence of a prior history of decompensated cirrhosis or a few weeks after the first episode of decompensation. Furthermore, ACLF is not a temporally-fixed syndrome; patients may progress or improve in a dynamic fashion. The ACLF mortality risk increases remarkably with the number of organs that fail; hence, several independent prognostic scores are used to better assess mortality in these patients[4,16,18-21].

INFLAMMATION IS A KEY FACTOR IN THE PATHOGENESIS OF DECOMPENSATED CIRRHOSIS AND ACLF

Systemic inflammation and immune system dysregulation are now proposed to integrate the main physiopathological pathway involved in the natural history of cirrhosis[5,6,22,23]. The recently-described CAID syndrome refers to the combination of immune deficiency and systemic inflammation that occurs as a consequence of persistent immune cell activation through infectious and non-infectious stimuli. These two components coexist in a dynamic manner from the initial to the final stages of cirrhosis, though in different

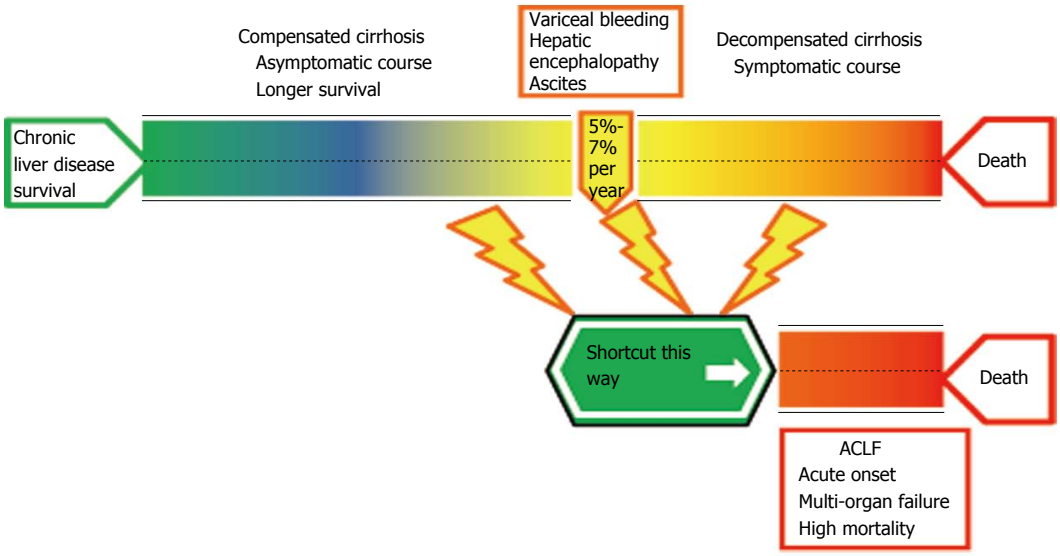

Figure 1 Natural history of cirrhosis. The classical compensated and decompensated phases of cirrhosis are divided by the presence of specific complications and marked by stable progression. A possible shortcut may occur after a decompensating event in any phase of cirrhosis, hastening the development of organ failure and a worse prognosis; this syndrome is termed acute-on-chronic liver failure (ACLF).

magnitudes along the way[3,6,23].

IMMUNE SYSTEM DAMAGE

The immune system alterations in cirrhosis are thought to be multifactorial and occur in a multi-step manner. The local injury takes place in the liver, where architectural disorganization caused by sinusoidal fibrosis impairs bacterial clearance[6,24]. Concomitantly, there is a diminished synthesis of innate immune system proteins and pattern recognition receptors (*i.e.*, Toll-like receptors) that, together, reduce the bactericidal capacity of the cells of the innate immune system (*e.g.*, stellate cells, neutrophils, natural killers, macrophages)[25,26]. As cirrhosis progresses, another key organ is affected: the gut. The gut-associated lymphoid tissue (GALT) is the first immunological barrier of defense against antigens and pathogens entering the organism from the intestine[27]. In advanced cirrhosis, the GALT is under the constant pressure of pathological bacterial translocation (BT) and bacterial products translocation that results from a leaky gut, an elevated enteric bacterial load, and changes in intestinal microbiota populations towards pathogenic species (dysbiosis)[22,28-34]. Finally, at a systemic level, immune cell function is compromised not only due to cytopenia, secondary to enlarged spleen sequestration when significant portal hypertension is present, but also affecting each cellular line individually[6,13]. In advanced cirrhosis, neutrophils have been shown to have deranged phagocytic activity of opsonized bacteria[35,36], as well as monocytes, that also exhibit impaired phagocytosis and diminished major histocompatibility complex class Ⅱ protein expression when located in ascitic fluid[37]. B lymphocytes show particular dysfunctions in their memory cell subset, and T lymphocytes display specific depletions of their helper and cytotoxic subsets[38,39]. These alterations become more significant as liver cirrhosis progresses. Eventually, the long-lasting activation of immune cells causes their exhaustion and reprogramming into a transient state of unresponsiveness to further bacterial product challenge; this phenomenon is termed "endotoxin tolerance"[6,22,23,40].

SYSTEMIC INFLAMMATION: THE GUILTY PARTY

Damage to the immune system is only one half of the problem. Systemic inflammation is mediated through the activation of all innate and adaptive immune cells, resulting in an increased production of pro-inflammatory cytokines and upregulated expression of cell activation markers[13,15,41,42]. In compensated cirrhosis, ligands released from necrotic hepatocytes, known as damage-associated molecular patterns (DAMPs), may activate the immune system and cause sterile systemic inflammation. In decompensated cirrhosis, other ligands also appear. Systemic inflammation is thought to be primarily triggered by BT or bacterial products (*e.g.*, lipopolysaccharide, methylated DNA) translocated from the intestinal lumen into the circulation. In this case, the culprits are termed pathogen-associated molecular patterns (PAMPs)[5,6,13,43]. At the decompensated stage, due to portal hypertension and the leaky gut, persistent BT further activates the immune system. In response to the continuous influx of PAMPs, the levels of pro-inflammatory cytokines and leukocyte activation antigens significantly increase[44-47]. Numerous cytokines and activation antigens are involved in this initial "pro-inflammatory" phenotype, such as tumor necrosis factor-α, interleukine-1 beta (IL-1β), IL-6, interferon-γ, IL-17, IL-18, ICAM-1, and VCAM-1. Concomitantly, the levels of anti-inflammatory cytokines (*e.g.*, IL-10,

Table 1 Proposed inflammation-related prognostic markers in advanced cirrhosis

Marker	Ref.	Prognostic implications	Study population	Limitations
SIRS	[52,53-55]	Portal hypertension-related complications and death	Decompensated cirrhosis patients admitted for acute decompensating events	Baseline elevated heart rate, respiratory frequency, and decreased PMN count in cirrhosis
Total leukocyte count	[16,48,56,57]	Development of ACLF, ACLF progression, ACLF related-mortality	ACLF	Hypersplenism possible cause of PMN count reduction, lack of clinically validated cut-off point
Absolute eosinophil count	[58]	Short-term mortality	Decompensated cirrhosis patients admitted for acute decompensating events	No external validation
Neutrophil: lymphocyte ratio	[59]	Short-term mortality	End-stage cirrhosis patients listed for liver transplant	No external validation
PCT	[62,63]	Infection, short-term mortality	Decompensated cirrhosis patients admitted for acute decompensating events	Lack of studies in non-infected cohorts
CRP	[62-68]	Infection, short-term mortality, HCC-related mortality	Decompensated cirrhosis patients admitted for acute decompensating events	Utility in organ allocation and HCC prognostic scores still to be validated

SIRS: Systemic inflammatory response syndrome; PMN: Polymorphonuclear leukocyte; ACLF: Acute-on-chronic liver failure; PCT: Procalcitonin; CRP: C-reactive protein; HCC: Hepatocellular carcinoma.

transforming growth factor-β) are decreased[3,6,42,43,48]. In the more advanced stages of cirrhosis, the immune system is exhausted and unable to mount functional innate and adaptive immune responses, resembling an endotoxin tolerance scenario. At this point, an "immunodeficient" phenotype is observed, characterized by increased levels of anti-inflammatory cytokines and leukocyte inhibitory antigens and deteriorated immune cell function[6,23,40,49]. An extreme version of this scenario has been suggested to be the underlying mechanism in ACLF, in which an immune-paresis state similar to sepsis occurs[49].

These clinical stages may have a gradual (decompensated cirrhosis) or abrupt (ACLF) onset and a dynamic evolution[6]. The excessive activation of the immune system may contribute to the symptoms of cirrhosis because systemic inflammation and oxidative stress, modulated by glutaminase gene alterations, have been described as the underlying mechanisms for hepatic encephalopathy[50,51]. A similar scenario has been proposed for ascites, since pro-inflammatory cytokines are responsible for the local release of nitric oxide and other vasodilators; this leads to the hyperdynamic circulatory state found in decompensated cirrhosis, effective hypovolemia, activation of the renin angiotensin system, and ultimately ascites formation[5,43]. In the absence of an acute superimposed injury, these events and the progressive impairment of left ventricular function[44,52] eventually lead to circulatory and renal dysfunction. Several studies have described renal damage to be mediated specifically by pro-inflammatory cytokines, PAMPs, and DAMPs, which reduce the glomerular filtration rate and damage tubular epithelial cells[4,16,53]. In ACLF the extreme manifestations of CAID are observed. The associated prognosis is directly related to the severity of systemic inflammation and the number of organ failures[3,16-18].

CLINICAL IMPLICATIONS OF SYSTEMIC INFLAMMATION IN CIRRHOSIS: IDENTIFICATION OF PROGNOSTIC FACTORS

Due to the overwhelming evidence implicating systemic inflammation in the natural history of cirrhosis, several easily available serum markers and clinical parameters have been proposed as prognostic tools to improve follow-up and management, especially in decompensated cirrhosis and ACLF. These markers are summarized in Table 1 and described further.

SIRS

SIRS is defined by the presence of at least two of the following criteria: altered body temperature (> 38 ℃ or < 36 ℃), elevated respiratory rate or hyperventilation (20 breaths/min or $PaCO_2$ < 32 mmHg), tachycardia (heart rate > 90 beats/min), and altered leukocyte count (> 12000/mm^3, < 4000/mm^3, or > 10% immature forms)[42]. The presence of SIRS has been associated with worse outcomes in the setting of decompensated cirrhosis. In a study evaluating a cohort of cirrhosis patients admitted for acute renal failure, the presence of SIRS was found to be a major independent prognostic factor, independent of infection[54]. The presence of SIRS was also found to predict the development of portal hypertension-related complications and death in cirrhosis patients having an episode of acute decompensation[55]. In two similar studies with larger cohorts, SIRS was found to be an independent predictor of poor outcome[56,57]. Hence, SIRS could be considered an additional prognostic factor for the severity of liver disease. Unfortunately, this syndrome can be difficult to assess in cirrhosis patients. Hypersplenism, hyperventilation associated

with encephalopathy, hyperkinetic circulatory syndrome, or the use of beta blockers may modify the clinical or biochemical parameters of SIRS[42]. New markers of SIRS that are less subject to heterogeneous findings would thus be particularly useful in cirrhosis.

LEUKOCYTE COUNT

Leukocyte count is an isolated element of SIRS frequently identified as a surrogate marker of this syndrome. In a large prospective observational study performed by the Chronic Liver Failure Consortium that aimed to describe the clinical features and prognostic factors of ACLF, leukocyte count was found to be an independent predictor of the development of ACLF, its severity, and its associated mortality[16,58]. In a large collaborative study in infected ACLF patients, leukocyte count was found to be an independent predictor of short-term mortality[59]. This finding has also been reported in ACLF patients without infectious decompensating events. In a study evaluating the relationship between portal hypertension and systemic inflammation in alcohol-related ACLF, disturbances in systemic and hepatic hemodynamics were associated with dysregulated inflammation, revealed by higher levels of leukocytes, C-reactive protein (CRP), and pro-inflammatory cytokines. These elevations, together with multi-organ failure and a marked activation of the sympathetic nervous system, were found to be predictors of higher mortality rates[51]. Leukocyte subsets and ratios have also been suggested as prognostic tools. In a study by Kotecha *et al*[60], that evaluated the role of absolute eosinophil count and procalcitonin (PCT) in predicting in-hospital mortality of admitted cirrhotic patients with SIRS, the baseline absolute eosinophil count of less than 104 cells/mm^3 accurately predicted in-hospital mortality in critically-ill cirrhosis patients with SIRS, independent of the MELD score or serum sodium levels. In addition, the neutrophil:lymphocyte ratio was described as an independent risk factor for death in a cohort of end-stage cirrhosis patients listed for liver transplantation[61]. Despite the fact that leukocyte count has been consistently defined as a risk factor for mortality in severely-ill cirrhosis patients, there are some drawbacks to this marker. One is the lack of a cut-off point for individual patient evaluation (*i.e.*, the specific mortality expected in an ACLF patient with a leukocyte count of 11000/mm^3 is unknown), diminishing its utility in everyday practice; such a cut-off point has only been determined for eosinophil count, without further external validation. In addition, the majority of these studies were conducted completely or partially in infected or alcohol-related ACLF patients, two etiologies associated with higher leukocyte counts *per se*, with only subgroup results available for the uninfected ACLF cohorts.

CRP AND PROCALCITONIN

Both serum proteins are tightly associated with SIRS. CRP, the prototype human acute phase protein, is a well-known marker of inflammation and is one of the most frequently-quantified molecules in clinical medicine[62]. CRP is synthesized mainly in the liver. CRP and PCT, a prohormone used as a marker of bacterial infections, is produced by most parenchymal tissues throughout the body during the acute phase of infection by these microorganisms[63]. Assays for CRP and PCT are readily-available, inexpensive, and more accurate than clinical parameters of SIRS for the identification of systemic inflammation. Both proteins have been evaluated as prognostic markers for short-term mortality in cirrhosis patients, usually in the context of infection[64,65]. However, CRP has also been suggested to be a useful tool independent of infection. In the prospective study by Cervoni *et al*[66] where the utility of CRP as a mortality risk factor in cirrhosis inpatients was evaluated, CRP levels $\geqslant$ 29 mg/L were found to be independent predictors of short-term mortality in cirrhosis patients with Child-Pugh scores $\geqslant$ B8, independent of age, MELD score, and co-morbidities; in this regard, CRP performed better than the presence of infection or SIRS. Di Martino *et al*[67] included CRP variation over 15 d as an additional element in the MELD score to better assess short-term mortality in decompensated cirrhosis patients. The inclusion of CRP improved MELD score accuracy in severely-ill cirrhosis patients admitted for acute decompensating events, but not in cirrhosis patients with planned admissions due to endoscopic procedures, *etc*[67]. Although CRP may be a useful addition to the MELD score in the setting of decompensated cirrhosis, several factors (*e.g.*, the usage of different CRP cut-off values according to the severity of cirrhosis and the need for two measurements of CRP in samples obtained 15 d apart) have reduced the utility of using CRP in organ allocation[68].

The use of CRP as a surrogate marker of survival has been studied in the setting of hepatocellular carcinoma (HCC). The presence of CRP levels > 6.3 mg/L, together with a neutrophil:lymphocyte ratio > 2.3, was identified as an independent risk factor for lower survival in HCC patients[69]. Similar findings were attained when CRP levels were compared to the levels of serum albumin: a CRP:albumin ratio of $\geqslant$ 0.037 was found to be an independent survival factor in HCC patients and correlated with tumor progression and reduced liver functional reserve[70]. Furthermore, it has been proposed that the addition of CRP to the currently-validated staging systems for HCC (*e.g.*, the Cancer Liver Italian Program, Japan Integrated Staging, Barcelona Clinic Liver Cancer classification system, Tokyo score, and tumor node metastasis classification) could improve their prognostic abilities[71].

FUTURE DIRECTIONS

The crucial role of systemic inflammation in the pathophysiology and prognosis of cirrhosis patients has been thoroughly described. Since SIRS is often difficult to

assess in cirrhosis patients, new objective, reproducible and readily-available surrogate markers are needed in order to optimize prognosis and lengthen survival. Leukocyte count, neutrophil:lymphocyte ratio, and absolute eosinophil count have been proposed, though with no clear cut-off points or extensive validation so far. PCT has also been suggested, yet its utility appears to apply exclusively to infected patients. CRP is useful as a prognostic marker in decompensated cirrhosis patients and ACLF despite the presence of infection, as well as in HCC. However, the value of adding CRP to current prognostic scores remains to be confirmed. Further basic research and prospective randomized studies that validate these and other markers are clearly warranted.

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EDITORIAL

Autophagy: A new therapeutic target for liver fibrosis

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Abstract

Hepatic fibrosis is a wound-healing response to liver injury and the result of imbalance of extracellular matrix (ECM) accumulation and degradation. The relentless production and progressive accumulation of ECM can lead to end-stage liver disease. Although significant progress has been achieved in elucidating the mechanisms of fibrogenesis, effective anti-fibrotic strategies are still lacking. Autophagy is an intracellular process of self-digestion of defective organelles to provide material recycling or energy for cell survival. Autophagy has been implicated in the pathophysiology of many human disorders including hepatic fibrosis. However, the exact relationships between autophagy and hepatic fibrosis are not totally clear and need further investigations. A new therapeutic target for liver fibrosis could be developed with a better understanding of autophagy.

Key words: Autophagy; Liver fibrosis; Hepatic stellate cells; Antifibrotic target

Core tip: Autophagy plays dual roles in hepatic fibrosis. On the one hand, it attenuates fibrosis by reducing hepatic injury *via* inhibiting inflammatory reaction and maintaining cellular homeostasis. On the other hand, it fuels activation of hepatic stellate cells (HSCs) by lipophagy and induces type I collagen synthesis. More studies using Atg selective knockdown mice or primary HSCs derived from Atg-deleted mice are needed. Selective inhibition of autophagy in HSCs is an attractive antifibrotic strategy.

Mao YQ, Fan XM. Autophagy: A new therapeutic target for liver fibrosis. *World J Hepatol* 2015; 7(16): 1982-1986 Available from: URL: http://www.wjgnet.com/1948-5182/full/v7/i16/1982.htm DOI: http://dx.doi.org/10.4254/wjh.v7.i16.1982

FIBROSIS

Liver fibrosis is a wound-healing response to a range of chronic liver diseases of different etiology, and drives

the progression of chronic hepatic diseases towards advanced liver cirrhosis and even hepatic carcinoma. Effective therapies are lacking besides diet control and physical exercise. Persisting parenchymal cell injury results in recruitment of immune cells, and activation and accumulation of fibrogenic cells. As the main source of liver fibrogenic cells, hepatic stellate cells (HSCs) lose cytoplasmic lipid droplets composed of retinyl esters to transdifferentiate from quiescent cells to activated myofibroblasts upon liver injury[1]. Myofibroblasts synthesize and secrete extracellular matrix (ECM) in an attempt to limit liver injury[2]. In addition, they also produce a wide range of matrix metalloproteinases (MMPs) that degrade ECM, and specific tissue inhibitors of metalloproteinase to inhibit activation of MMPs[3]. In brief, hepatocyte injury, immune cell recruitment, and fibrogenic cell activation contribute to the imbalance of ECM accumulation and degradation, which ultimately lead to fibrosis.

AUTOPHAGY

Autophagy is a catabolic intracellular pathway, targeting defective or excessive organelles to the lysosomes for degradation into amino acids, free fatty acids or other small molecules used for material recycling or energy harvest. Autophagy, usually stimulated by energy restriction, stress or inflammation, is regarded as a survival mechanism that plays a critical role in maintaining cellular homeostasis, which is involved in many human disorders including fibrotic disease. Three different kinds of autophagy are defined based on how the substrates are delivered to the lysosomes for degradation: macroautophagy, microautophagy, and chaperone-mediated autophagy, with macroautophagy being the major type. Although it is regarded as a cell-protective mechanism, excessive autophagy can cause cell death, known as type Ⅱ programmed cell death[4]. However, it is unclear whether autophagy directly executes cell death or is a secondary effect of apoptosis. Autophagy can be considered a double-edged sword[5], and more investigations are needed to explore the complicated roles of autophagy.

AUTOPHAGY IN FIBROSIS: "HERO" OR "VILLAIN"?

Autophagy reduces fibrosis by hepatocyte injury attenuation

An increasing body of evidence supports the notion that autophagy participates in the pathophysiology of many human disorders including hepatic fibrosis. However, whether it is a hero or villain in hepatic fibrosis is still controversial.

Recent studies have demonstrated that autophagy impairment results in liver disease exacerbation due to reduction of degradation of defective organelles and unfolded proteins, which causes oxidative and endoplasmic reticulum stress[6-9] (Figure 1). Autophagy is increased in mice treated acutely with alcohol, in parallel with a marked reduction of serum inflammatory markers and tissue triglyceride level[10]. Autophagy may degrade activated caspase-8, a death receptor[11], thus exhibiting an antifibrotic effect by limiting liver injury. Furthermore, in α-1 antitrypsin (AT) deficiency, a disease in which the α-1 AT mutant Z protein results in protein aggregation and chronic liver injury, an autophagy-enhancing drug was demonstrated to reduce the hepatic load and reversed fibrosis[12]. Collectively, all the studies consistently supported that autophagy acted as a hero in hepatic fibrosis.

Controversial issues of autophagy and HSC activation

It had been unclear whether autophagy participates in HSC activation until the study of Zhu *et al*[13] in 1999, which demonstrated that rapamycin, a known immunosuppressive agent, inhibited HSC proliferation and limited fibrogenesis in mouse models treated with carbon tetrachloride (CCl_4). They further demonstrated that rapamycin decreased HSC proliferation. As an immunosuppressant, rapamycin inhibited growth factor signaling in nonimmune as well as immune cells[14], which may largely explain its antifibrotic effect. The authors pointed out that mammalian target of rapamycin (mTOR) negatively regulated autophagy. The binding of rapamycin and mTOR appeared to block interleukin-2-dependent proliferation of T cells and even other cells[14]. Similar results were gained in another two studies[15,16]. However, it is unfortunate that no one has detected any change in autophagy during improvement of fibrosis, because rapamycin or its analogs stimulate autophagy by inhibiting mTOR. The antifibrotic effect of rapamycin depends on its antiproliferative effect on fibrotic cells or the indirect effect of autophagy stimulation remains unclear.

Fortunately, 10 years later, another study discovered that autophagic flux was increased during HSC activation and was inhibited by bafilomycin A1, an autophagy inhibitor. HSC activation was blocked by 3-methyladenine (MA) or chloroquine, suggesting that inhibition of HSC activation could be achieved by interruption of different phases of autophagy[17]. This evidence strongly indicates that autophagy is involved in HSC activation (Figure 1). Another discovery that merits further consideration is that platelet-derived growth factor BB, which activates HSCs, stimulates the location of microtubule associated protein light chain 3 Ⅱ, an important biomarker protein of autophagy and lipid droplets, implying a potent relationship between HSC activation and lipid metabolism.

Hernández-Gea *et al*[18] have shown that autophagy releases lipid that promotes fibrogenesis by activating HSCs in mice and in human tissues. Inhibition of autophagy by pharmacological antagonism or Atg5 and Atg7 knockdown in mice also resulted in attenuation of fibrogenesis, as well as increased lipid content in stellate

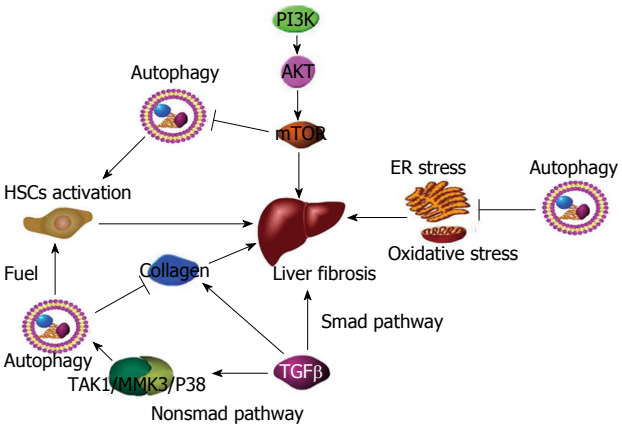

Figure 1 Mechanisms involved in autophagy and fibrosis. (1) Phosphoinositide 3-kinase promotes phosphorylation of AKT, which subsequently leads to stimulation of mTOR and inhibition of autophagy. mTOR activation promotes hepatic fibrosis; (2) Autophagy fuels HSC activation, leading to hepatic fibrosis; (3) TGF-β promotes collagen synthesis and fibrosis *via* the Smad pathway. Furthermore, TGF-β stimulates autophagy *via* the non-Smad TAK1/MMK3/P38 pathway, leading to collagen degradation and fibrosis reduction; and (4) Autophagy attenuating ER stress and oxidative stress, and ultimately reduces fibrosis. mTOR: Mammalian target of rapamycin; HSC: Hepatic stellate cells; TGF-β: Transforming growth factor-β; TAK1/MMK3/P38: TGF-β-activated kinase 1-MAPK kinase 3-p38.

cells isolated from Atg7F/F mice[18]. Likewise, HSC-specific deletion of Atg7 in mice which were treated with either CCl_4 or thioacetamide, also lead to obvious reduction of tissue fibrosis with preserved intracellular lipid droplets[19]. These results strongly support that autophagy induces tissue fibrogenesis by degradation of intracellular lipid droplets, which is known as lipophagy. Autophagy is a generalized feature of fibrotic cells, and a similar phenomenon is not only observed in the liver, but also in other organs such as the kidneys and lungs[19].

Autophagy and transforming growth factor-β1 related signaling pathways

Transforming growth factor (TGF)-β1 is a classical signaling pathway in liver fibrosis and induces autophagy[20], suggesting that autophagy participates in fibrosis *via* the TGF-β pathway. TGF-β1 may stimulate autophagy *via* the TGF-β-activated kinase (TAK)1-MAPK kinase (MKK)3-p38 and TAK1-AMP-activated protein kinase (AMPK) pathways, leading to profibrotic responses[21] (Figure 1). However, it is plausible that TGF-β acts as both an apoptosis promoter and suppressor, which may relate to its regulation of autophagy, and plays dual roles in apoptosis[21]. TGF-β protects glomerular mesangial cells from apoptosis during serum deprivation *via* autophagy induction[22]. Moreover, TGF-β is involved in ECM synthesis and degradation. One study showed that TGF-β induced autophagy in MMC *via* the TAK1-MKK3-p38 signaling pathway, and autophagy promoted intracellular degradation of collagen (Figure 1). The dual functions of TGF-β as both an inducer of collagen synthesis and an inducer of autophagy and collagen degradation underscore the multifunctional nature of TGF-β[23].

Autophagy and collagen degradation

Genetic and pharmacological inhibition of autophagy in mice resulted in increased levels of type Ⅰ collagen in mouse kidneys and primary mesangial cells, suggesting that autophagy promotes intracellular degradation of type Ⅰ collagen, which is a major component of ECM[23]. Autophagy attenuates endoplasmic reticulum stress by eliminating misfolded procollagen[24]. Furthermore, Beta (2)-adrenergic stimulation triggers autophagy in cardiac fibroblasts and promotes intracellular collagen degradation and inhibits cardiac fibrosis[25]. However, this effect has been demonstrated in other organs, and whether it exhibits the same effect in liver remains unclear.

The above studies marked a milestone in the exploration of the role of autophagy in hepatic fibrosis. Autophagy is mostly a cell survival mechanism that attenuates hepatic inflammatory injury and ultimately inhibits liver fibrosis. Given more insight into the role of autophagy in HSC activation, we have realized a new perspective that autophagy is responsible for activation of HSCs and other hepatic fibrogenic cells, by intracellular lipid degradation, leading to fibrosis. TGF-β induces autophagy, therefore, its role in liver fibrosis needs further investigation. We have to take into account that although autophagy may be a critical pathway in ameliorating hepatic injury in the short term, its long-term effect in fibrogenic cells may worsen chronic liver diseases, which could be regarded as a side effect in antifibrotic therapy[19].

We suggest that if autophagy could be selectively inhibited in HSCs and other fibrotic cells, autophagy special blocker would be an attractive candidate of antifibrotic strategies. Nonetheless, inhibition of cell-specific autophagy is exciting, yet more challenging in a tissue containing various types of cells. Further research is needed, targeting different receptors on the cell surface that may activate different effect of autophagy. It would also be useful to determine whether HSC activation is completely blocked by autophagy inhibition or just partly reversed to a quiescent phase, and the appropriate extent and time of autophagy should be seriously considered. Several genes participate in induction of autophagy. This raises the question of whether there is a link between autophagy and HSC phenotypic transformation.

Controversial issues of autophagy and mTOR

The data from Thoen *et al*[26] seem to contradict the HSC-activating yet autophagy-inhibiting effect of mTOR[27], because mTOR contributes to cell proliferation, including HSCs[13,28]. Likewise, it has been demonstrated that rapamycin, an mTOR target inhibitor and autophagy stimulator, reduces liver fibrosis[13,14,16], which is contradicted by later studies showing that autophagy induces HSC activation. Liu *et al*[29] have indicated that autophagy inhibitor 3-MA significantly

inhibits proliferation and activation of HSCs by arresting the cells in G2 phase. Whether autophagy inhibits or promotes HSC proliferation is controversial. Whether the inhibitory effect on proliferation of fibrogenic cells depends on mTOR inhibition itself or an indirect action on autophagy remains unclear. In a recent study, TGF-β rapidly activated its canonical Smad signaling pathway, and recruited a noncanonical pathway involving mTOR kinase to induce matrix protein collagen Ⅰ expression, thus inducing fibrosis[30]. Therefore, it is essential to investigate the relationship among mTOR, autophagy, and HSC proliferation. Few studies have focused on lipid metabolism and HSC activation, leaving the mechanism of intracellular lipid degradation poorly understood. More research, especially with selective knockdown of Atg in mice, or HSCs derived from Atg-deleted mice, will shed light on this[26].

Finally, we hypothesize that since fibrosis is the result of imbalances of ECM accumulation and degradation, could it be a new direction to focus on the translocation of ECM turning into cell from extracellular matrix. Then intracellular matrix could be enclosed by autophagosome and subsequently fuses with lysosome to be degraded. Since autophagy has been demonstrated to promote the degradation of type Ⅰ collagen in kidney, some level of autophagy may help in treatment of hepatic fibrosis.

CONCLUSION

Autophagy is a novel target playing dual roles in human diseases including liver fibrosis. Autophagy may help cells to live through stress conditions and attenuate inflammation, leading to fibrosis reduction. Autophagy is involved in collagen degradation, which may contribute to fibrosis attenuation. However, autophagy fuels HSCs to be activated and promote fibrosis. The effect of autophagy on liver fibrosis is complex and still controversial. With a better understanding of the effects of autophagy on hepatic fibrosis, autophagy may have potential as a target of antifibrotic therapy.

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REVIEW

Focal liver lesions: Practical magnetic resonance imaging approach

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Abstract

With the widespread of cross-sectional imaging, a growth of incidentally detected focal liver lesions (FLL) has been observed. A reliable detection and characterization of FLL is critical for optimal patient management. Maximizing accuracy of imaging in the context of FLL is paramount in avoiding unnecessary biopsies, which may result in post-procedural complications. A tremendous development of new imaging techniques has taken place during these last years. Nowadays, Magnetic resonance imaging (MRI) plays a key role in management of liver lesions, using a radiation-free technique and a safe contrast agent profile. MRI plays a key role in the non-invasive correct characterization of FLL. MRI is capable of providing comprehensive and highly accurate diagnostic information, with the additional advantage of lack of harmful ionizing radiation. These properties make MRI the mainstay for the noninvasive evaluation of focal liver lesions. In this paper we review the state-of-the-art MRI liver protocol, briefly discussing different sequence types, the unique characteristics of imaging non-cooperative patients and discuss the role of hepatocyte-specific contrast agents. A review of the imaging features of the most common benign and malignant FLL is presented, supplemented by a schematic representation of a simplistic practical approach on MRI.

Key words: Malignant; Benign; Magnetic resonance imaging; Focal liver lesions; Hepatobiliary contrast agents

Core tip: With the widespread of cross-sectional imaging, a growth of incidentally detected focal liver lesions (FLL) has been observed. A reliable detection and characterization of FLL is critical for optimal patient management. Magnetic resonance imaging

(MRI) plays a key role in non-invasive characterization of FLL. The multiparametric ability of pre- and post-contrast sequences is an intrinsic advantage of MRI to reach an accurate diagnosis. New techniques such as diffusion-weighted sequences and hepatocyte-specific contrast agents are being currently used in clinical practice, which might further improve the detection and characterization of FLL.

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INTRODUCTION

With the widespread of cross-sectional imaging, a growth in rate of incidentally detected focal liver lesions (FLL) has been observed. A reliable detection and characterization of FLL is critical for optimal patient management. The majority of FLL arising in noncirrhotic liver are benign[1], even in patients with known extra-hepatic malignancy. Cysts, hemangiomas, focal nodular hyperplasias (FNH), and hepatocellular adenomas (HCA) are the most commonly encountered benign lesions[1-5]. The most commonly encountered malignant lesions in noncirrhotic liver are metastases[6-8]. Hepatocellular carcinomas (HCC), and to a lesser extent intrahepatic cholangiocarcinomas (IHC), occur mainly in the setting of chronic liver disease, and represent the most common primary liver malignancies[7,9-14].

A tremendous development of new imaging techniques has taken place during these last years. Maximizing accuracy of imaging in the context of FLL is paramount in avoiding unnecessary biopsies, which may result in post-procedural complications up to 6.4%, and mortality up to 0.1%[15-17]. Nowadays, magnetic resonance plays a key role in management of liver lesions, using a radiation-free technique and a safe contrast agent profile[18,19].

The heightened soft-tissue resolution and sensitivity to intravenous contrast agents provided by magnetic resonance imaging (MRI) makes it an invaluable problem-solving tool for fully characterizing FLL[20,21]. Previous studies estimated the sensitivity and specificity of MRI for the diagnosis of FLL of 94% and 82%-89%, respectively[22].

This review focuses on the diagnostic performance of MRI in evaluating the most common benign and malignant FLL. As a summary, a practical educational approach to FLL on MRI is also presented.

MRI PROTOCOL

With the current state of the art technology, magnets of 1.5 Tesla (T) and 3T field strength are considered the standard of reference in providing high-quality and consistent MR images. Giant advances in MRI have been achieved in the last decade in regards to each of the following: hardware (high-performance gradient coils and phased-array surface coils), software (new sequence design and new parallel imaging technology and acceleration techniques), and contrast agents (hepatocyte-specific agents) have made a major impact on imaging of the liver.

In our perspective, an adequate imaging protocol has to be short, comprehensive, and standardized to allow reproducibility and consistency of image quality and diagnostic performance. A comprehensive protocol allows the evaluation of the parenchyma, vasculature, and biliary system; by using either breathing-independent sequences or breath-hold sequences that minimize motion artifact and spatial misregistration. Gradient-echo (GRE) sequences generally are used in T1-weighted sequences and fast spinecho sequences are used in T2-weighted sequences[20,23].

The state of the art MRI protocols rely on a combination of fat-suppressed and non-fat-suppressed T2-weighted images (T2-WI), in- and opposed-phase (IP/OP) T1-WI and dynamic pre- and post-contrast fat-suppressed T1-WI[7,12].

The predominant information provided by T2-WI is about fluid content, fibrotic tissue and iron content (reflected by high, low, and very low signal intensity, respectively). Fat suppression is generally applied for at least one set of images in order to increase lesions conspicuity.

Pre-contrast T1-WIs are extremely important in lesion characterization. Most FLL are mildly or moderately low in signal intensity. Lesions with high-fluid or fibrous tissue content are moderately or substantially low in signal intensity. Hemorrhagic lesions, and those with high protein or fat content, are high in signal intensity on T1-WI. Fat suppression technique facilitates reliable characterization of fatty lesions. GRE sequences provide T1-WI in a short amount of time and allow chemical shift imaging in a single breath-hold (dual echo acquisition). The two echo times are chosen so that fat and water peaks are IP and OP, respectively. OP images are useful to detect small amounts of intracellular fat in liver lesions and in hepatic parenchyma. If fat and water are in the same voxel, the signal intensity decreases on the OP images, with maximal signal loss occurring when fat and water are in equal proportion.

Gadolinium-enhanced images are performed routinely in a multiphasic dynamic fashion, using three-dimensional (3D) fat-suppressed GRE breath-hold T1-WI. The acquired phases include late arterial, portal venous, interstitial, and delayed phases; which allow the assessment of enhancement kinetics (a reflection of both vascularity and permeability). However, most diagnostic information can be derived from the late hepatic arterial phase, also called hepatic-arterial dominant phase, with the correct timing characterized by observing contrast enhancement in the portal vein branches and no enhancement in the hepatic veins[24]. Suggested

Table 1 Comparison between extracellular and hepatocyte-specific agents (Multihance® and Eovist®)

	Extracellular contrast agents	Hepatocyte-specific agents	
		Multihance®	Eovist®
Advantages	Robust arterial and portal venous phase imaging	Robust arterial and portal venous phase imaging	Hepatobiliary imaging
	Price and availability	Hepatobiliary imaging	Short delay for hepatobiliary phase (20 min)
		Smaller dose administration	Smaller dose administration
		Safer for renal impaired patients	Safer for renal impaired patients
		Price	
Disadvantages	No hepatobiliary phase	Availability (not available in all countries)	Less robust arterial and portal venous phase imaging
	NSF cases reported with less stable agents	Longer delay for hepatobiliary phase (90-180 min)	Pitfalls for inexperienced readers
			Price

methods for ensuring an optimal arterial phase liver MRI have varied between empiric fixed delay or individually-tailored timing. The latter method is recognizable to be more accurate and with improved reproducibility, and involves either pre-scanning with a test bolus, or tracking bolus arrival in the descending aorta[25]. In our practice, a bolus-tracking technique (CARE bolus software) is employed to capture the late hepatic arterial phase. This is performed by the technologist, who, when visualizing maximum aortic enhancement at the level of celiac trunk, will provide an 8 s breath-holding instructions prior to initiating the scan.

Adequate delay between initiation of contrast injection and initiation of the sequence is crucial to aid in optimizing the detection of hypervascular lesions. The precision in timing the portal venous phase is more flexible and less critical (45-75 s), characterized by enhancement of the entire hepatic vascular system. The portal venous phase maximizes contrast between hypovascular lesions and the background liver, and can be used to evaluate the contrast washout pattern, which is a useful discriminating feature. Images acquired 1.5 to 10 min after contrast injection are in the interstitial/delayed phase of enhancement, which aid in evaluating persistent enhancement in hemangiomas, washout in HCC, or delayed enhancement of fibrotic tissue or tumors, such as in cases like cholangiocarcinoma.

Intravenous MR contrast agents can be divided into extracellular (ECA) and hepatocyte-specific agents (HSA). ECA equilibrate with the extracellular fluid space after intravenous injection and are excreted by glomerular filtration, similar to computed tomography (CT) agents. This permits multi-phase dynamic post-contrast imaging as described earlier.

Like ECA, HSA allow the multi-phase dynamic post-contrast imaging. Moreover, they show some degree of biliary excretion, allowing a late hepatobiliary phase acquisition. Due to the action of known cellular membrane transporters, only normal functioning hepatocytes take up HSA and excrete them to the biliary tree[26]. Hepatobiliary phase images are easy to recognize because both the liver and the bile ducts are markedly enhanced. The blood vessels as well as all non-hepatocellular lesions and lesions with impaired hepatocytes all appear hypointense.

The two HSA available are Eovist® (gadoxetic acid, Bayer Health-Care Pharmaceuticals, marketed as Primovist® outside the United States) and MultiHance® (gadobenate dimeglumine, Bracco). With Eovist®, 50% of the dose is taken up by hepatocytes and eliminated by biliary excretion, compared to 3%-5% with MultiHance®. Hepatobiliary phase images are acquired 20-40 min after Eovist® injection, compared to 1.5-3 h after MultiHance® injection. Advantages and disadvantages of these agents and extracellular contrast agents are shown in Table 1.

The substantial difference on the biliary elimination of Eovist® compared to the other contrast agents affects the classical MR technique, lesion appearance, and thus image interpretation[27]. These differences provide not only advantages on detection and lesion characterization, but also new pitfalls in imaging interpretation[27]. The advantages in the evaluation of FLLs are appreciated in the distinction between FNH and HCA, and in the diagnosis of HCC and metastasis; while the pitfalls are related to the less favorable behavior as an extracellular agent and the "pseudowashout" of benign lesions[27]. Washout is historically linked to malignant lesions, particularly to hypervascular metastases and HCC. While using Eovist®, the "pseudowashout" may pose a risk in the diagnosis of benign lesions. As an example, hemangiomas are classically described as hypervascular lesions with centripetal fill-in, sustained in late dynamic postcontrast phases. With Eovist®, the accumulation of contrast can be masked by the intense hepatic parenchymal enhancement, giving the "illusion" of washout of the hemangioma[27].

At our center, we use MultiHance® as the standard contrast agent since it shows better enhancement on dynamic evaluation[26,28], and reserve Eovist® for selected cases on a problem-solving basis.

Recently, diffusion-weighted imaging (DWI) sequences have been shown to be an emerging contributor for liver MRI[29-32] and are being incorporated in most abdominal MR protocols. Diffusion is a physical process of random movement of water molecules. This movement of intracellular water molecules is restricted by the presence of cell membranes. In highly cellular tissues, such as neoplasms, diffusion is restricted due to the

relative larger intracellular volume and high density of cellular membranes. DWI exploits this phenomenon and its image contrast is based on differences in the mobility of water protons (as a measure of cellularity), between different tissues[31]. This MR technique should be used in combination with conventional unenhanced and contrast-enhanced MRI. It is especially useful in patients with contraindication to gadolinium contrast agents[32].

Additional sequences may be added to the protocol on specific clinical settings. For diffuse deposition diseases, fat-quantification or T2 star (T2*) sequences can be added.

Despite all development in MRI, its diagnostic performance is still affected by motion artifacts, which may result in inconsistent image quality. Motion artifacts, especially those produced by physiological motion caused by patient respiration, may distinctly degrade the quality of MR images. In patients who are unable to cooperate with breath-hold instructions, the sequences that are more affected are the T1-weighted GRE sequences. In order to minimize these artifacts, new motion-robust sequences have been implemented. The magnetization-prepared rapid gradient-echo (MP-RAGE) sequence is a 2D, single-section acquisition technique that can be used to obtain motion-free and moderate quality images. The acquisition times per-section are as short as 1 s[33]. These sequences can be used pre- and post-contrast. Additionally, a recently described new application of MP-RAGE IP/OP images is able to replace standard dual-echo chemical shift imaging with moderate to good image quality[34]. Recent developments in MR data sampling and *k*-space filling have been used to acquire 3D-GRE T1-WI. Radial data sampling 3D GRE can be used as a free-breathing sequence, providing high-quality pre- and post-contrast images[2,34,35]. The major drawback of radial 3D-GRE is the long acquisition time and therefore low temporal resolution of this technique. Until now, conventional radial 3D-GRE sequence is unable to provide, in a consistently fashion, critical scanning phases, *i.e.,* the late hepatic arterial phase. New sequences are being developed in order to provide a compromise between spatial and temporal high-resolution images, with reduced acquisition time and increased motion resistance.

BENIGN LESION

Hemangiomas

Hemangiomas are tumors of mesenchymal origin and are the most common benign liver solid lesions. The prevalence of these lesions ranges from 1%-20%, more frequently between fourth and fifth decade of life, showing a female predilection (ratio of 2-5:1)[36-38]. The size of hemangiomas usually remains stable and can vary from a few millimeters to more than 20 cm[38]. Complications are rare, and large lesions may become symptomatic due to compression of adjacent structures, rupture, or spontaneous hemorrhage[13].

On MRI, the majority of hepatic hemangiomas display pathognomonic pre- and post-contrast imaging features (Figure 1) enabling a correct diagnosis with high accuracy[39,40]. Frequently, hemangiomas show moderately high signal intensity on T2-WI, usually less bright than simple cysts or cerebrospinal fluid, and low signal intensity on T1-WI[1,41]. On post-contrast images, a nodular or "flame-shaped", discontinuous, peripheral enhancement is observed, as well as late, progressive, centripetal filling, and persistent delayed enhancement, similar to that of hepatic vessels. Larger hemangiomas may show incomplete filing along the dynamic imaging due to central scarring. Conversely, small (< 2 cm) hemangiomas may show rapid complete filling on the late arterial phase images. Subcentimeter hemangiomas may fade to isointensity to the background liver parenchyma in interstitial phase images; however, they never washout. On the arterial phase, especially the small rapidly enhancing subcapsular hemangiomas may show a perilesional enhancement[40], and this finding should not be regarded as an atypical or suspicious feature. Nevertheless, hemangiomas may uncommonly show atypical morphologic characteristics. The possible atypical findings are scarring, septations, capsular retraction, calcifications, cystic transformation and fluid-fluid level. Extremely rare is the interval growth, imposing the exclusion of other diagnostic possibilities, namely malignancy[14,38,41]. The end stage of a hemangioma involution results in appearance of hyalinized or sclerosed hemangiomas. At this point these lesions lose the homogeneous high signal intensity on T2-WI and the typical globular enhancement may not be seen[38,41]. As stated above, it should be emphasized that, when using Eovist®, small rapid filling hemangiomas will not show the typical features described earlier during the late interstitial phase due to the rapid uptake of contrast by the background liver parenchyma, giving the illusion of washout. Furthermore, hemangiomas will not show any uptake on the hepatobiliary phase, and will show the same appearance as non-hepatocytes containing lesions, *e.g.*, cysts, metastasis, and to a lesser extent HCA[28,38].

Focal nodular hyperplasia

FNH consists of a non-encapsulated lesion composed by non-neoplastic hepatocytes in a disorganized array, surrounding a central fibrous scar with a dystrophic arterial vessel[5,7,42]. There is some histopathologic heterogeneity of FNHs, with uncommon "non-classical" histologic subtypes described in the literature[42,43]. FNH is the second most common solid benign FLL. Most often found in young and middle-aged patients, FNH has a clear female predominance (8-12:1 ratio)[1,42].

MRI is considered the best imaging tool for FNH diagnosis, with a sensitivity of 70% and a specificity of 98%[44]. For some authors, contrast-enhanced MRI is considered the gold standard[5]. As FNH are composed of hepatocytes, they are barely discernable from normal parenchyma on precontrast images, appearing iso- or hypointense on T1-WI, and iso- or slightly hyperintense

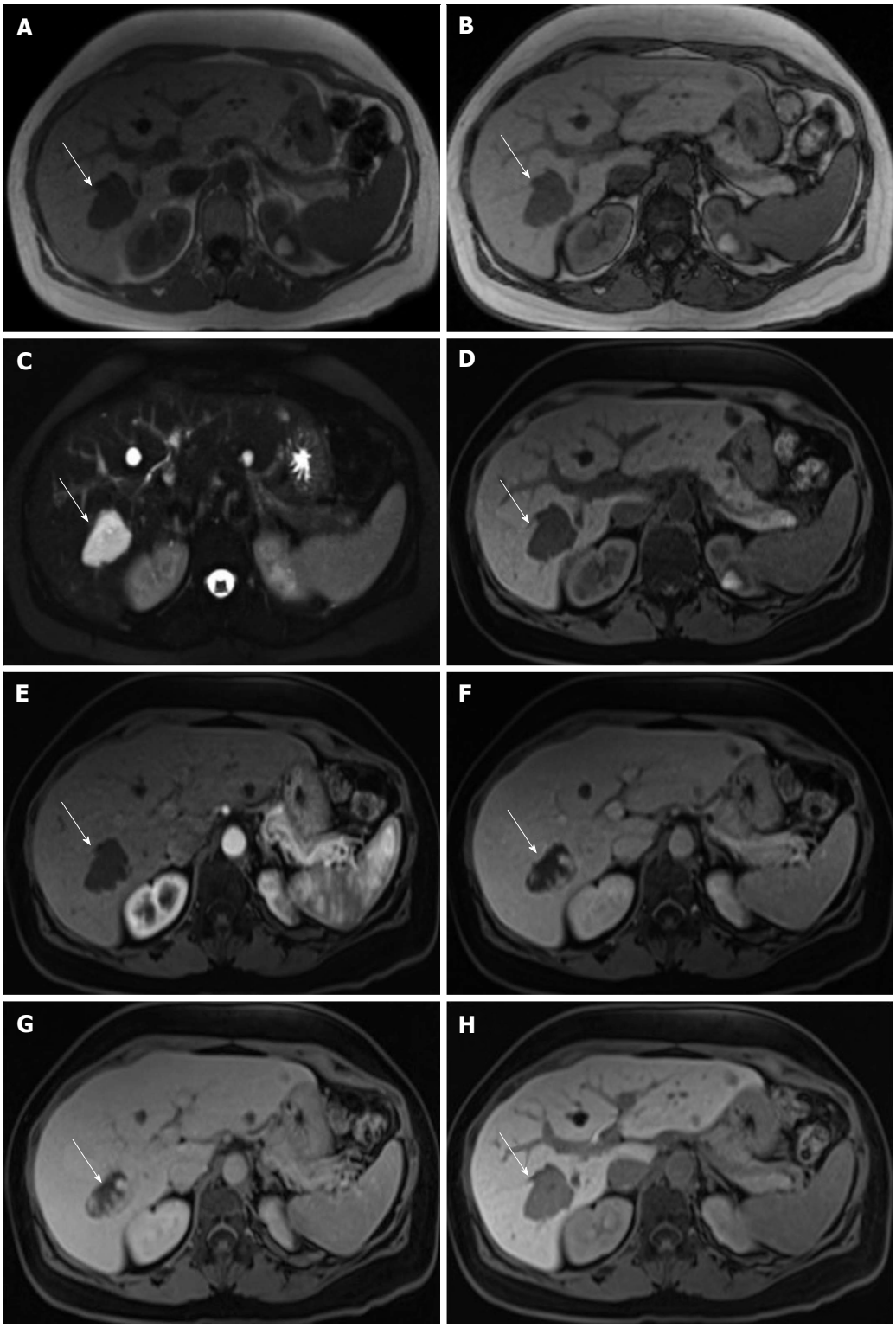

Figure 1 Hemangioma. In (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and post hepatocyte-specific contrast agent (Eovist®) fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F), interstitial (G) and hepatobiliary (H) phases. There is a lobulated lesion on the right hepatic lobe (arrows), showing marked low signal intensity on T1-WI (A, B and D) and marked high signal intensity on T2-WI (C). The lesion demonstrates peripheral and discontinuous nodular enhancement (E), which become larger and coalescent on delayed postcontrast images (F and G), showing a progressive centripetal filling. Due to the absence of hepatocytes, hemangiomas show low signal intensity on the hepatobiliary phase (H), acquired 20 min after the administration of the hepatocyte-specific contrast agent. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

on T2-WI. In approximately 50%-84% of cases, the central scar can be seen with low signal intensity on T1-WI and moderate high signal on T2-WI[42,44]. On postcontrast images, FNHs show typical enhancement pattern: early arterial homogeneous enhancement, which becomes isointense to the background liver on portal venous phase, and late enhancement of the central scar (Figure 2). No washout is seen with FNH[43,44].

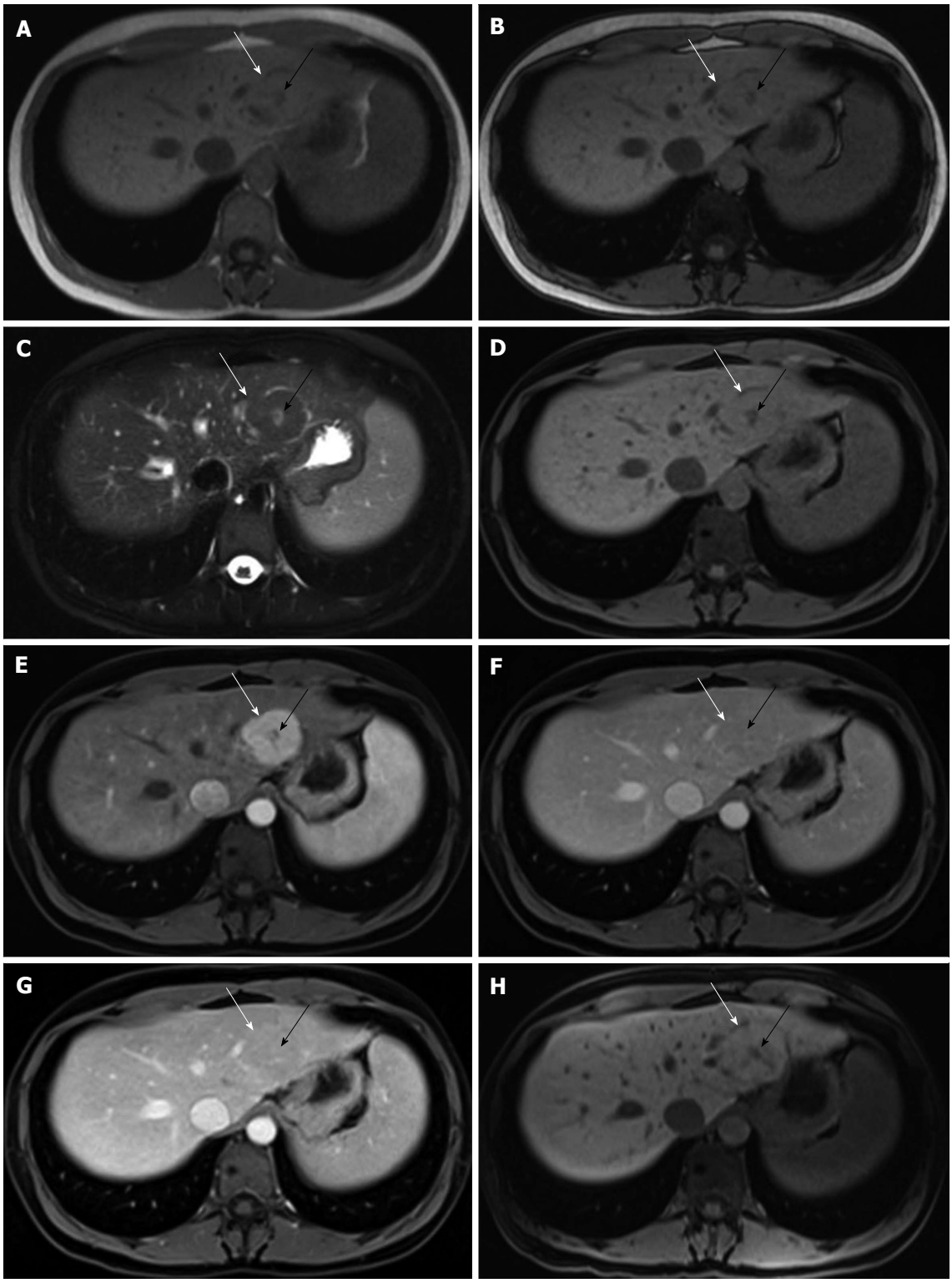

Figure 2 Focal nodular hyperplasia. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and post hepatocyte-specific contrast agent (Eovist®) fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F), interstitial (G) and hepatobiliary (H) phases. There is a lesion on the left hepatic lobe (white arrow, A-H), showing isointense signal comparing to the surrounding liver on non-contrast T1-WI (A, B and D) and on T2-WI (C). The lesion also shows a central scar (black arrow, A-H), which is hypointense on T1-WI (A, B and D) and hyperintense on T2-WI (C). The lesion demonstrates homogeneous enhancement on early post-contrast images (E), becoming isointense to the underlying liver parenchyma (F and G). The progressive enhancement of the central scar is depicted on the delayed post-contrast images (G). On the hepatobiliary phase, 20 min after the administration the hepatocyte-specific contrast agent, the lesion shows uptake of the contrast agent, becoming minimally hyperintense comparing to the surrounding liver parenchyma. Since the central scar has no hepatocytes, there is no uptake of the contrast agent, becoming hypointense comparing to the liver and to the rest of the lesion. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

The prevalence of typical features of FNH in literature ranges from 22%-70%[44], mainly related to different study designs and histopathologic heterogeneity of these lesions[43]. Using HSAs, the presence of normal

functioning hepatocytes can be demonstrated. Unlike the majority of HCA, FNHs show, on the vast majority of cases, iso- or hyper-enhancement on the hepatobiliary phase[1,7,45].

HCA

HCA is an uncommon benign primary FLL, and most often encountered in women of childbearing age taking oral contraceptives. Unlike FNH, HCAs are true neoplasms, defined as the monoclonal proliferation of well-differentiated hepatocytes arranged in sheets and cords. They lack portal triads and interlobular bile ducts. Nowadays, according to their genotypic and phenotypic characteristics HCAs are classified into 3 major molecular subtypes[5,46]: (1) inflammatory (formerly known as telangiectatic FNHs); (2) hepatocyte nuclear factor 1-alpha (HNF-1α) inactivated; and (3) Beta (β)-catenin-activated lesions. A fourth group can be considered including those HCAs that are unclassified in the previous subtypes. As different groups have distinct probability for HCC transformation, a pre-operative diagnosis is ideal for an appropriate patient management.

Generally, on MRI HCAs show mild to moderate high signal intensity on T2-WI and enhancement on the late arterial phase on post-contrast sequences (Figure 3). HCA do not show uptake on hepatobiliary phase with HSA. Although further validation is required, specific MRI features can be used to identify HCA subgroups[47-49]. Inflammatory HCAs (50%) tend to show peripheral marked high signal on T2-WI and maintained enhancement on more delayed images[1,47,49]. HNF-1α-inactivated HCAs (35%-45%) show diffuse intralesional fat deposition, responsible for higher signal intensity on non-fat-suppressed T1-WI and drop of signal on OP images. β-catenin-activated HCAs (10%-15%) findings are less defined, showing vaguely defined scars or poorly defined areas of high signal on T2-WI. For the first two subgroups (majority of adenomas), MRI have specificities ranging from 88%-100%[3,49]. These HCAs have null or extremely low probability of HCC transformation. β-catenin-activated HCAs have a high probability of malignancy transformation[5,46,48].

Sometimes MRI features of HCAs can overlap with FNHs. Distinctive features should be stressed. HCAs rarely show a central scar and much more frequently depict intralesional fat[44]. Homogeneity strongly suggests FNH over HCA[44]. The utilization of HSA is recommended to help in the distinction between FNH and HCA. The former showing increased uptake on hepatobiliary phase, while HCAs, usually, show no enhancement[4,28,50].

Benign cystic lesions

Cystic benign liver lesions are common and may represent a broad spectrum of entities ranging from developmental cysts to neoplastic lesions. It is important to distinguish them from malignant lesions that can show cystic transformation (as metastases or hepatocellular carcinoma). Fluid-containing benign liver lesions can be grouped broadly into simple or complex cysts[51].

The differential diagnosis of simple cysts includes benign developmental hepatic cysts, biliary hamartomas, foregut cysts, Caroli disease, and adult polycystic liver disease[51]. The benign developmental hepatic cyst shows homogeneously low signal on T1-WI and homogeneously strong high fluid signal on T2-WI (Figure 4). The margins are well defined and no enhancement is shown on postcontrast sequences. Biliary hamartomas are usually small (< 1.5 cm), round or irregular, and may show very thin and uniform peripheral enhancement (Figure 5), due to compressed liver parenchyma[52,53]. They have no connection to the biliary tree. Conversely, in Caroli disease the varying size cysts communicate with the biliary tree. Communication with the biliary system can be further confirmed using dedicated cholangiographic sequences or HSAs. The cysts depicted on adult polycystic liver disease appear as benign developmental hepatic cysts. MRI is the best modality for identifying cysts complicated by hemorrhage or infection[52].

Benign complex cysts are traumatic, inflammatory, or neoplastic in nature. Traumatic cystic lesion may occur after blunt or penetrating trauma, or iatrogenic injury, such as after cholecystectomy or liver surgery. On MRI, bilomas and seromas may resemble simple cysts, while hematomas show different intensity based on the age of the blood products[52].

The most frequent inflammatory cystic lesions are abscesses and hydatid cysts. With the advent of effective antimicrobial therapy, currently biliary tract pathologies have surpassed portal seeding from appendicitis and diverticulitis, as the most common source of pyogenic liver abscesses[54]. Abscesses are thick-walled lesions with low signal intensity on T1-WI and high signal intensity on T2-WI, with progressive enhancement of the wall[1]. Adjacent parenchyma shows high signal on T2-WI (edema) and enhancement on the arterial phase, due to inflammatory reaction[1,52,55] (Figure 6). Hydatid cysts are due to Echinococcus infestation. On MRI, daughter cysts and internal septa are readily visualized on T2-WI. Most echinococcal cysts show variable low to high signal intensity on T1-WI, depending on the amount of proteinaceous debris, and markedly high signal on T2-WI. The fibrotic component and the presence of calcifications appear as a hypointense pericystic rim, on both T1- and T2-WI. Cyst walls and internal septa enhance on post-contrast images[55].

Although rare, biliary cystadenoma (BCA) is the most frequent benign cystic neoplasm. It has biliary origin, with most of them (85%) arising from the intrahepatic bile ducts[1,55]. BCA usually appear as large (mean 12 cm; range 3-40 cm), well-defined and multi-loculated intrahepatic cyst. On MRI, BCAs typically show high signal on T2-WI and show variable T1 signal intensity due to proteinaceous content or blood products. Septal or mural calcifications (depicted as signal voids on all sequences) and fluid-fluid levels are occasionally seen. Post-contrast sequences may demonstrate enhancement

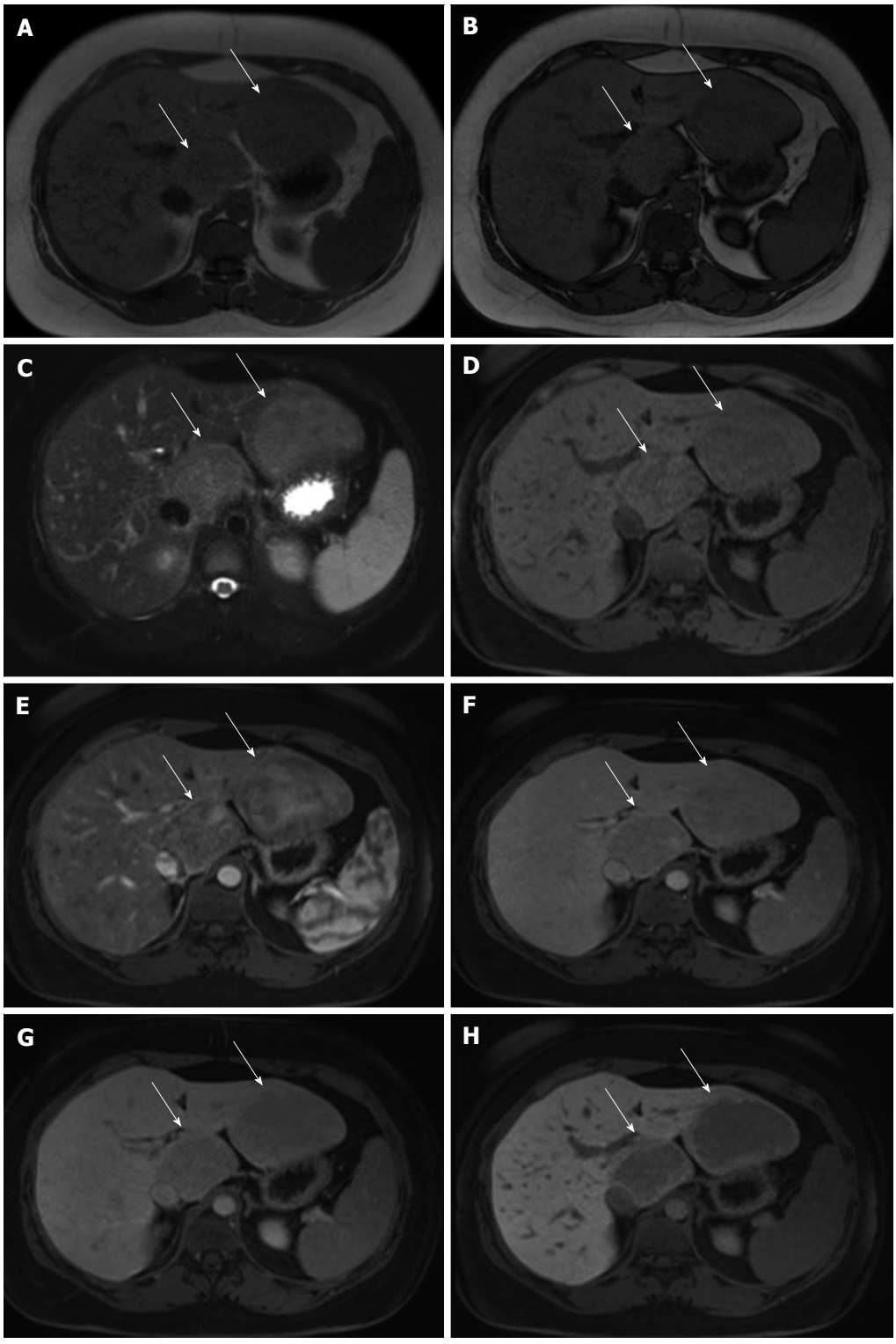

Figure 3 Hepatocellular adenomas. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and post hepatocyte-specific contrast agent (Eovist®) fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F), interstitial (G) and hepatobiliary (H) phases. Two focal liver lesions are noted on the left and caudate lobes (arrows, A-G) of a noncirrhotic liver, showing slight drop of signal intensity on opposed-phase (B) comparing with the in-phase (A) T1-WI, which is related to minimally fat content. Note that the liver parenchyma also shows minimal steatosis. The lesions demonstrate mild high signal intensity on T2-WI (C), heterogeneous enhancement on the postcontrast arterial phase image (E) and subsequent washout on later postcontrast images (F and G). On the hepatobiliary phase, acquired 20 min after the hepatocyte-specific contrast agent, the lesions show no contrast uptake, excluding the diagnosis of FNHs. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images; FNH: Focal nodular hyperplasias.

of the capsule, septa, and any mural nodules[1,55]. Biliary cystadenocarcinoma can develop from a BCA. It can be difficult to differentiate BCA and biliary cystadenocarcinoma preoperatively, but this is usually unnecessary on short-term, as both require complete surgical excision[1,52].

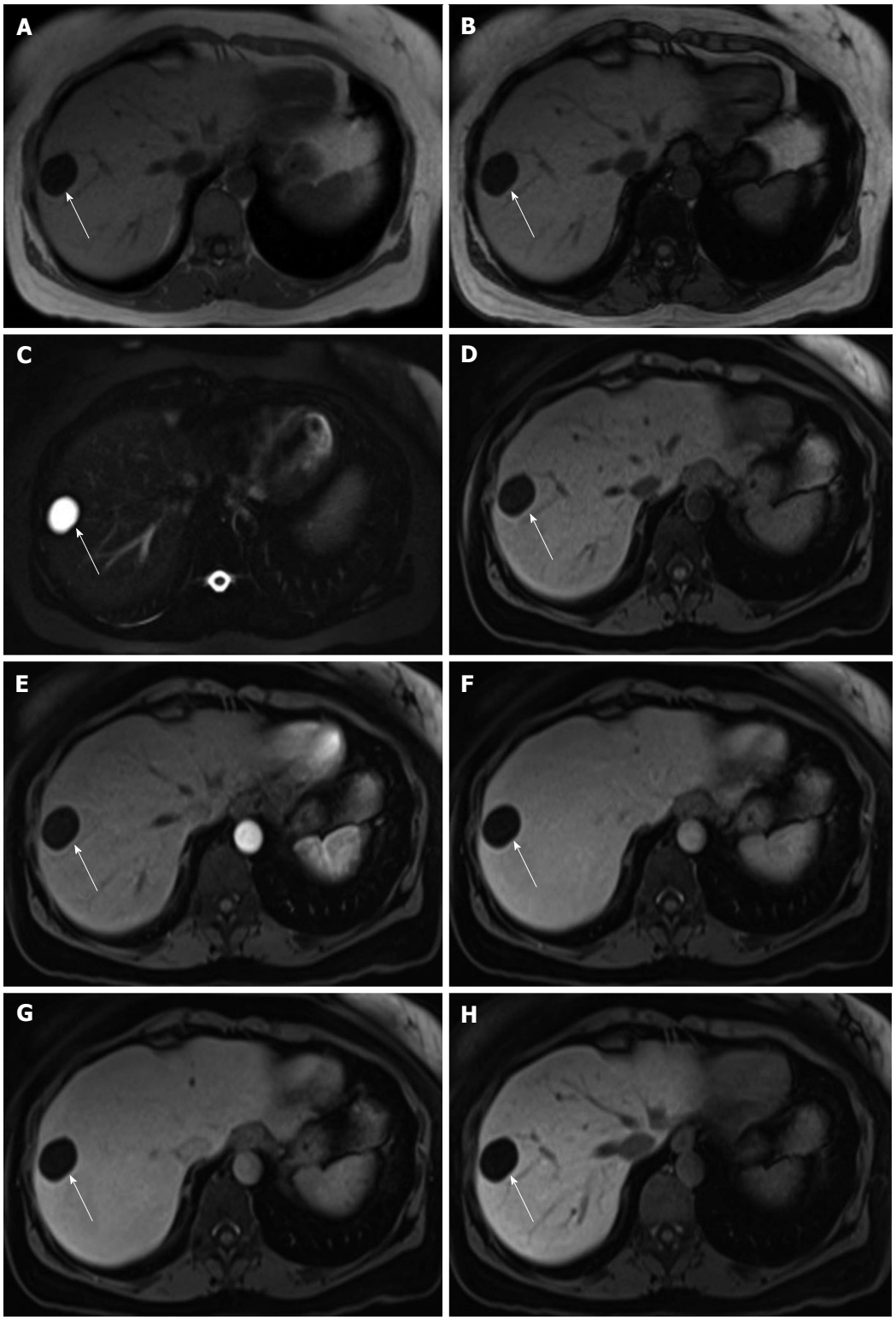

Figure 4 Cyst. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and post hepatocyte-specific contrast agent (Eovist®) fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F), interstitial (G) and hepatobiliary (H) phases. There is a well-defined lesion on the right hepatic lobe (arrow, A-H) showing marked homogeneous low signal intensity on T1-WI (A, B and D), homogeneous very high signal intensity on T2-WI (C) and no enhancement after gadolinium administration (E-H), consistent with simple liver cyst. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

MALIGNANT LESIONS

HCC

HCC is a malignant neoplasm with hepatocellular origin. It is the most common primary malignancy of the liver and it occurs almost exclusively in the context of chronic liver disease (CLD) and liver cirrhosis[56]. HCC has been proved to develop by multistep carcinogenesis from a low grade dysplastic nodules to an overt HCC, in a progressive dedifferentiation and neoangiogenesis phenomena[56-58].

MRI plays a pivotal role in the detection and char-

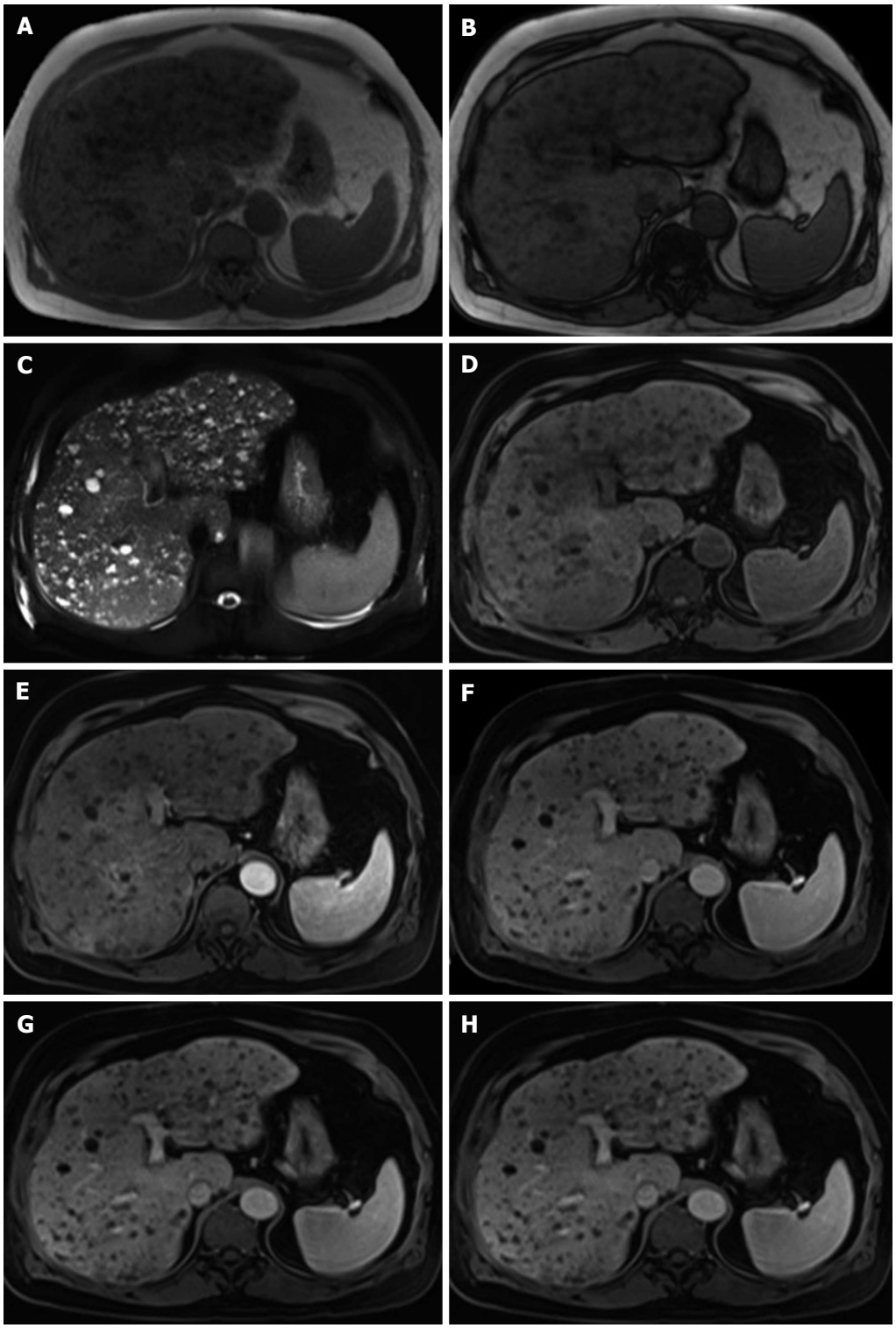

Figure 5 Multiple biliary hamartomas (also known as von Meyenburg complex). In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and postcontrast fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F) and interstitial (G) phases. There are multiple well-defined lesions scattered throughout the liver, smaller than 1.5 cm each. The lesions show low signal intensity on T1-WI (A, B and D), high signal intensity on T2-WI (C) and no enhancement after gadolinium administration (E-G). A thin peripheral enhancement is often present due to compressed liver parenchyma. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

acterization of HCC, with estimated sensitivity and specificity of 97.4% and 100%, respectively[59,60]. Even for HCC with a size < 2 cm, MRI have a good sensitivity, estimated to 82.6%[60]. This is particularly important since successful treatment of HCC is dependent on early detection and diagnosis[59,61].

In the context of CLD, classic MRI findings of HCC include slightly low signal intensity on T1-WI, slightly high signal intensity on T2-WI, increased heterogeneous arterial enhancement, and washout with fibrous tumor

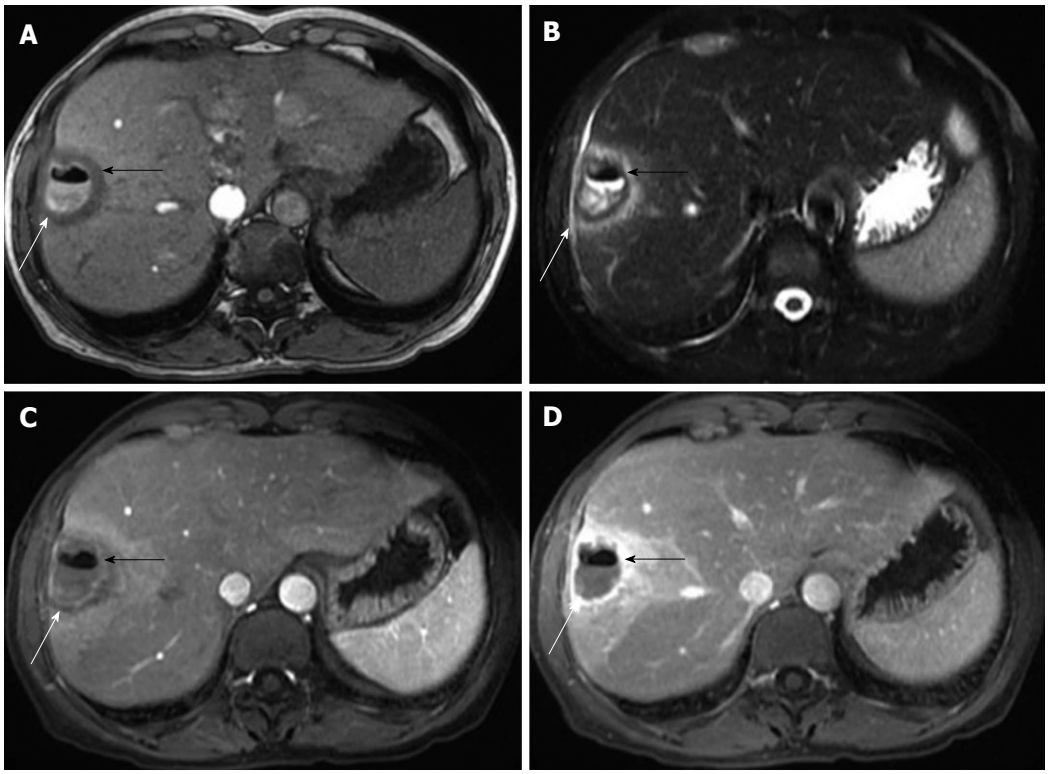

Figure 6 Abscess. GRE T1-WI (A), fat-suppressed FSE T2-WI (B), and postcontrast fat-suppressed 3D-GRE T1-WI at the arterial (C) and portal venous (D) phases. A thick-walled oval shaped lesion is present on the right hepatic lobe (white arrow, A-D), showing an air/fluid level content (black arrow, A-D). There is an associated halo of edema surrounding the lesion, showing low signal intensity on T1-WI (A), high signal intensity on T2-WI (B) and marked enhancement after gadolinium administration (C and D), which is consistent with active inflammation. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

capsule enhancement on the delayed phase[62] (Figure 7).

The 2011 recommendations by the Association for the Study of Liver Diseases state that a diagnosis of HCC is made if a nodule larger than 1 cm is depicted on MRI (or multi-detector computed tomography), showing arterial enhancement and subsequent "washout" during portal venous or equilibrium phases[63]. These guidelines criteria show a lower sensitivity and specificity for small HCCs (< 2 cm)[64-66]. In order to improve the accuracy of MRI on small HCC, other parameters may be used in conjunction with the dynamic post-contrast sequences, namely high signal on DWI or T2-WI[64,67]. With HSA, most HCC lesions show prominent hypointensity compared to the hyperintense background liver parenchyma because of the absence of normal functional hepatocytes[67]. Although, uncommonly, well-differentiated HCCs may show some enhancement on the hepatobiliary phase[12,68,69]. The combination of routine dynamic and hepatobiliary imaging has been reported to be both sensitive and specific for HCC (sensitivity 67%-97%, specificity 83%-98%)[69,70-77]. Two recent meta-analyses found a pooled sensitivity of 91% and specificity of 93%[78,79].

In the context of CLD and cirrhosis, the distinction between benign and precursor lesions (regenerative and dysplastic nodules, respectively) and HCCs is of the utmost importance[80]. Although they can present with higher T1 signal intensity compared to background liver tissue, regenerative nodules are often indistinct on T1- and T2-WI. The dynamic post-contrast imaging show the same signal as the background parenchyma throughout all phases[12,14]. Dysplastic nodules show histological characteristics of abnormal growth caused by genetic alteration and are classified accordingly to the level of dysplasia, on low- and high-grade dysplastic nodules[14]. On MRI, low-grade dysplastic nodules are often indistinctive from regenerative nodules, and radiologists reserve that terminology for lesions larger than 2 cm in size[12,81]. High-grade dysplastic nodules show iso to high signal on T1-WI and iso signal intensity on T2-WI. They may show intense early enhancement and fade to isointensity, but do not show washout[12,81]. As opposed to HCC, regenerative and low-grade dysplastic nodules show iso-enhancement to the surrounding liver parenchyma. Patients with the diagnosis of high-grade dysplastic nodules are at higher risk of developing HCC and should have closer follow-up MRI.

IHC

IHC is the second most common primary hepatic malignancy, accounting for 10%-20% of all primary liver malignancies[82-84]. Background of CLD such as cholangitis, viral hepatitis (especially hepatitis C) and liver cirrhosis, are known specific risk factors[84]. Primary sclerosing cholangitis is the most well known of these conditions. In terms of growth characteristics, cholangiocarcinomas may be mass-forming, periductal-infiltrating, or

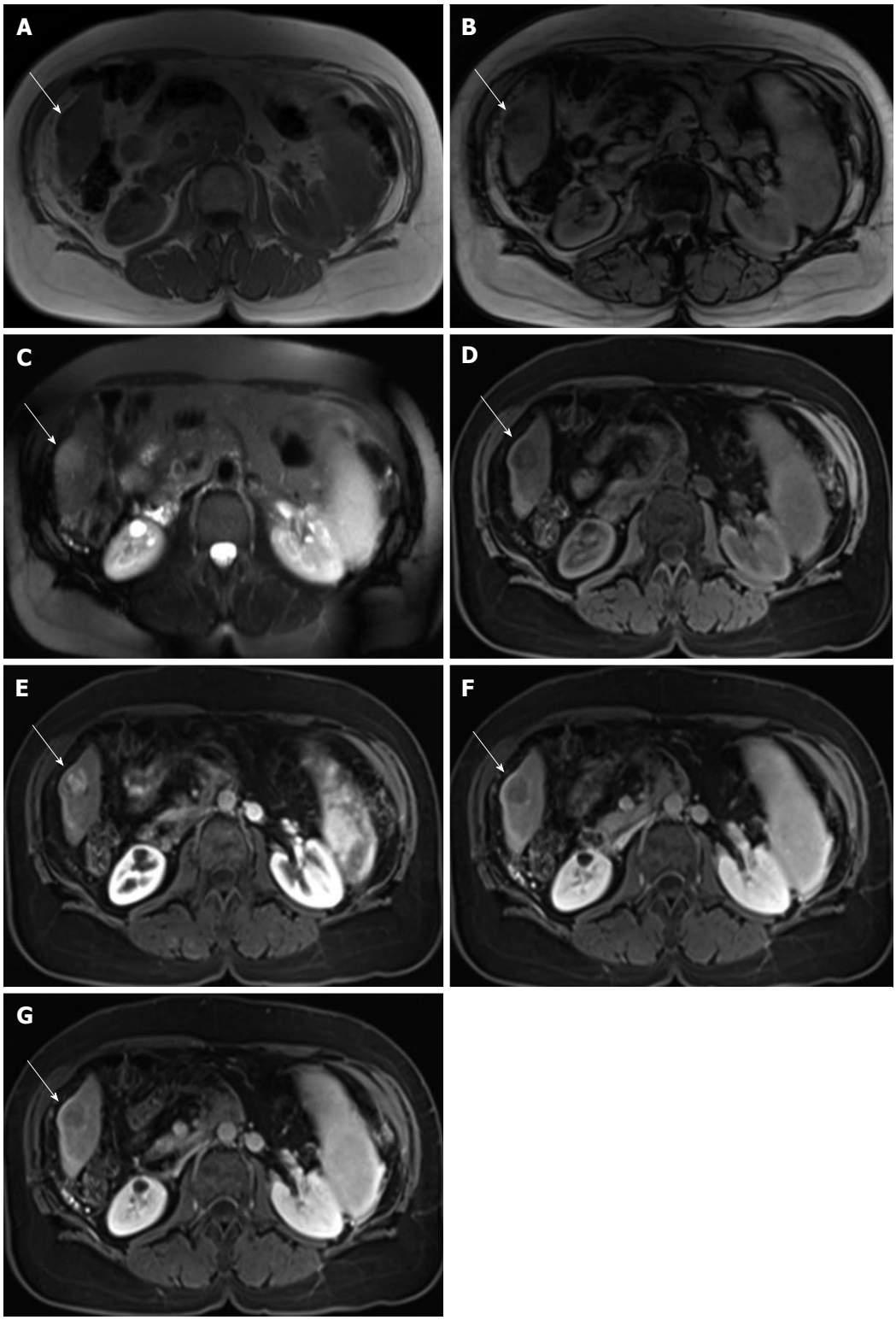

Figure 7 Hepatocellular carcinoma. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and postcontrast fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F) and interstitial (G) phases. There is a small peripheral lesion on the right lobe of a cirrhotic liver (arrow, A-G), showing drop of signal intensity on opposed-phase (B) comparing with the in-phase (A) images, which is suggestive of fat content. The lesion demonstrates mild high signal intensity on T2-WI (C), low signal intensity on pre-contrast T1-WI (C), heterogeneous enhancement on early post-contrast (E) and subsequent washout with associated pseudocapsule on delayed post-contrast images (F and G), in keeping with a small fat-containing hepatocellular carcinoma. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

intraductal. Mass-forming cholangiocarcinoma is the most common IHC, accounting for the majority of IHC, and are defined as a rounded mass located in the liver parenchyma[83,84].

MRI allows the distinction between IHC and HCC with high degree of confidence. At present, MRI with

MR cholangiopancreatography has become the imaging modality of choice for diagnosis and staging of cholangiocarcinoma, with the similar accuracy of computed tomography combined with direct cholangiography[85]. The MR appearance of IHC depends on the proportion of fibrosis, necrosis and mucin. Typically they show low to iso-signal intensity on T1-WI, and variably high signal intensity on T2-WI[82,83]. Capsular retraction is sometimes described, reflecting the desmoplastic nature of the tumor. In some cases vascular encasement and dilated bile ducts peripheral to the mass may be seen. Early continuous rim enhancement followed by progressive heterogeneous enhancement of the remainder of the lesion is often seen (Figure 8). The late enhancement is due to the fibrotic nature of cholangiocarcinoma. Near one third of cholangiocarcinomas are hypervascular. Nanashima *et al*[86], Kim *et al*[87] and Al Ansari *et al*[84] reported that 46%, 29%, and 28%, respectively, of the intra-hepatic cholangiocarcinomas in their studies showed hypervascular enhancement pattern. This appearance is well recognized and might carry a better prognosis with longer disease-free survival[87,88]. Conversely, IHC in patients with chronic viral hepatitis or cirrhosis tend to be very hypervascular[86,89]. Xu *et al*[90] showed that the density of arteries and micro-vessels of IHC in a cirrhotic liver was higher than that in IHC without underlying cirrhosis and comparable to that in cholangiocarcinoma component of combined HCC-IHC. This vascular difference in IHC may be responsible for the hypervascular enhancement of IHC in the context of cirrhosis[90].

When using HSA, mass-forming IHC may have a pseudowashout pattern with Eovist®-enhanced MR images because of progressive background liver enhancement and no enhancement of the IHC[91]. Hepatobiliary imaging with Eovist®, showed increased lesion conspicuity and better delineation of secondary nodules and intrahepatic metastasis, which may aid the evaluation of IHC[92]. Further, it has been suggested that it can be helpful for therapy planning due to the exact depiction of the tumor borders[93].

Satellite lesions are markedly more conspicuous on hepatobiliary phase of Eovist®-enhanced MRI, proposing a potential role for hepatobiliary MR agents in evaluation of the tumor resectability. In a study by Kim *et al*[87], 93% of mass-forming intra-hepatic cholangiocarcinoma exhibited a special pattern of enhancement in hepatobiliary phase of Eovist®-enhanced MRI, described as cloud-like hyperintensity in the central portion of the tumors, surrounded with a low signal intensity rim, which appears as a defect in the vicinity of the hyperintense normal liver parenchyma.

Metastasis

Metastasis is the most common liver malignancy, outnumbering primary liver malignant neoplasms with a ratio of 40:1. Moreover, it has been shown that 40% of patients with extrahepatic malignancy show liver metastasis at the autopsy. Accurate detection and characterization of liver metastasis is critical in patient management, namely in determining treatment and prognosis[26]. MR is rapidly evolving as the primary imaging modality for the detection and characterization of liver lesions including metastases in many centers.

On MRI, hepatic metastases have variable appearances depending on the primary tumor. Generally, metastases show mild to moderate high signal intensity on T2-WI and low signal intensity on precontrast T1-WI. Cystic metastases and those with necrosis show increased T2 signal (more common in neuroendocrine tumors, sarcomas, and melanoma metastases). A subset of liver metastases shows T1 hyperintensity for a variety of reasons. One subset is the fat-containing metastases, which are easily characterized by the drop of signal on OP and/or fat-suppressed sequences. Other subset is metastases that contain paramagnetic substances such as melanin, extracellular methemoglobin and protein. A good example is melanoma metastases, which are often T1 hyperintense because of their melanocytic content and/or occasional hemorrhage. On dynamic post-contrast imaging, metastases are characterized as hypervascular, iso-vascular, or hypovascular, when they show more, similar, or less enhancement compared to the background liver parenchyma, respectively, at the late arterial hepatic dominant phase (Figure 9).

Most of liver metastases are from extra-hepatic adenocarcinomas and generally they tend to be hypovascular[82]. Hypervascular metastases are usually seen in neuroendocrine tumors, renal cell, thyroid and breast carcinoma, melanoma, and sarcoma.

HSAs and DWI are useful for detection of small hepatic metastases, demonstrating improved sensitivity over conventional T2-WI MR techniques and, significantly increased sensitivity compared to CT imaging[27,94-96]. As metastatic tumors do not contain functioning hepatocytes, they appear hypointense during the hepatocellular phase, resulting in a high contrast between enhancing liver tissue and metastases[26]. Metastases show high signal on DWI and may increase the confidence of the diagnosis[94,95]. The combination of HSAs and DWI yield better diagnostic accuracy and sensitivity in the detection of small liver metastasis than each magnetic resonance scan sequence alone. In one study, the combined set showed significantly improved sensitivity (mean values, 97.5%/95.0% on per-lesion/per-patient basis) than each imaging set alone (mean, 90.7%/83.7% for Eovist® set, and 91.6%/83.0% for DWI set) on both per-lesion basis and per-patient basis[97].

While the use of MRI on detection and characterization of liver metastatic disease is well established, the role on the assessment of treatment response is less defined[21]. It has been suggested the benefits of MRI over CT[98-100]. This is evident in the setting of pseudoprogression diagnosed on CT due to high density of hemorrhagic treated lesions and bevacizumab-containing chemotherapy regimens on metastatic colorectal cancer[98-100]. However, more studies are needed in order

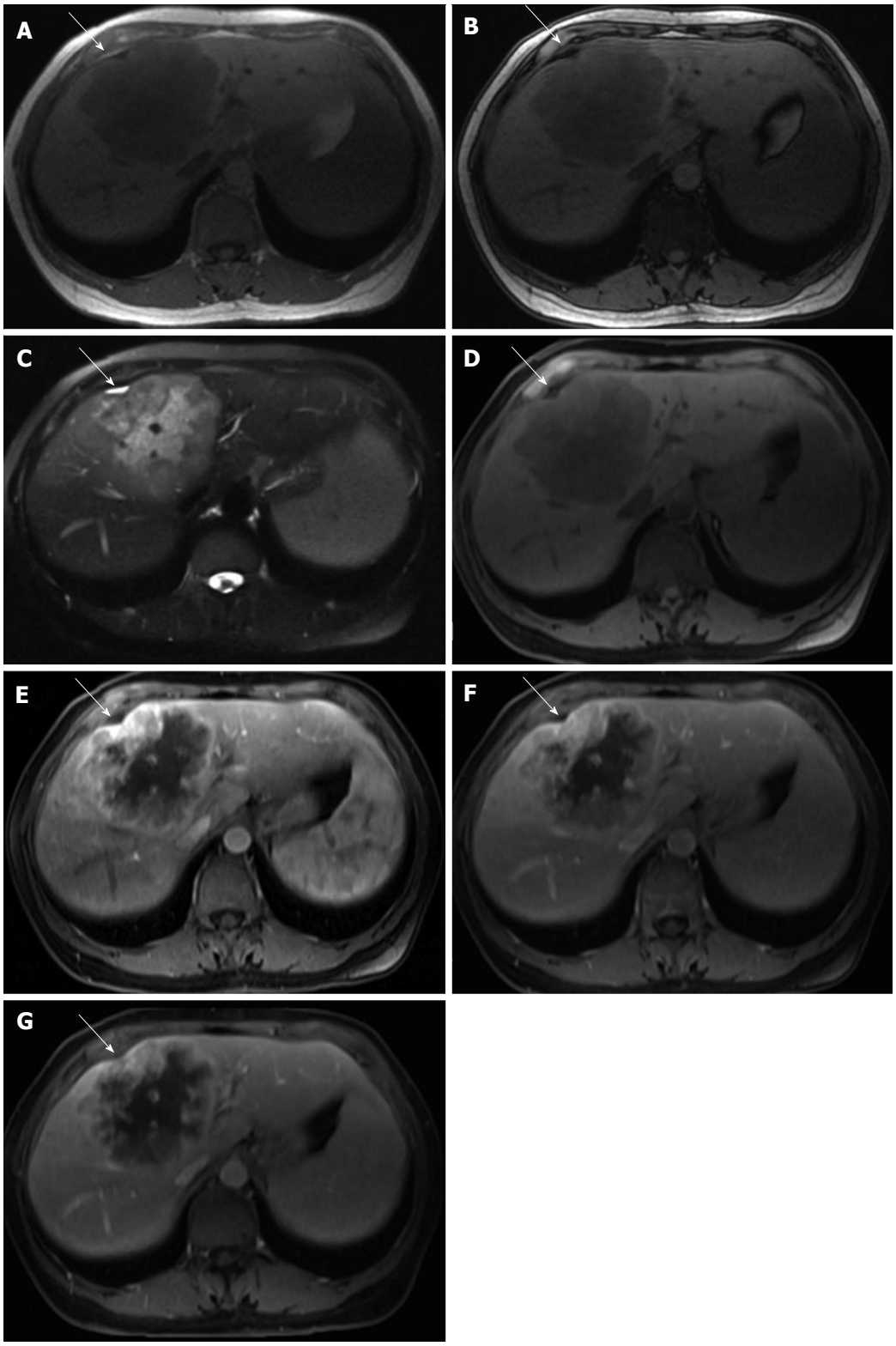

Figure 8 Intrahepatic cholangiocarcinoma. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and postcontrast fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F) and interstitial (G) phases. The tumor shows low signal intensity on T1-WI (A, B and D), high signal intensity on T2-WI (C), and heterogeneous peripheral continuous and progressive enhancement on postgadolinium images (E-G). Associated capsular retraction is also noted (white arrow, A-G). GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

to fully comprehend the role of MRI on the assessment of treatment response of metastatic liver lesions.

Simplified practical approach

MRI is a highly sensitive and accurate modality for the characterization of FLL. Although many hepatic lesions have characteristic imaging features, the interpretation should rely on a combination of lesion assessment, background liver assessment, and clinical parameters. For liver lesion characterization, HSA are

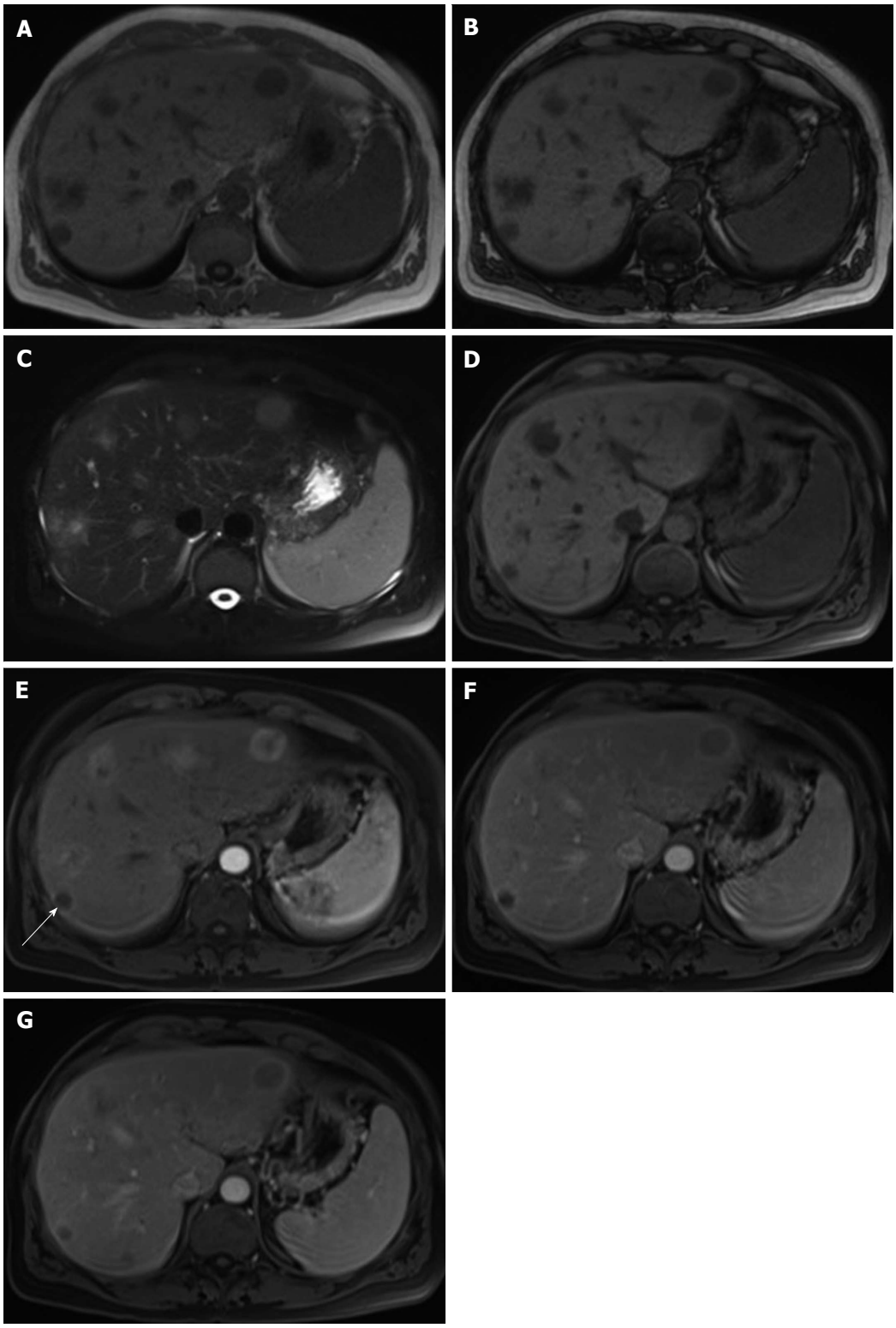

Figure 9 Metastases. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and postcontrast fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F) and interstitial (G) phases. Multiple metastases are present throughout the liver, showing low signal intensity on T1-WI (A, B and D) and high signal intensity on T2-WI. Most of these lesions show hypervascular characteristics, while one in segment VII (arrow, E) shows ring enhancement. Late washout is perceived, a feature that is characteristic of carcinoid metastases (G). GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

used to assess the presence of intralesional functional hepatocytes. DWI is also useful in differentiating benign from malignant lesions. Although clinical utility has been proven, further investigation is needed to better delineate the role of HSA and DWI in characterizing FLL. A simplified schematic representation of the typical imaging features of the most common benign and malignant hepatic lesions is provided in Figures 10 and 11.

The hepatocellular nature of a lesion can usually

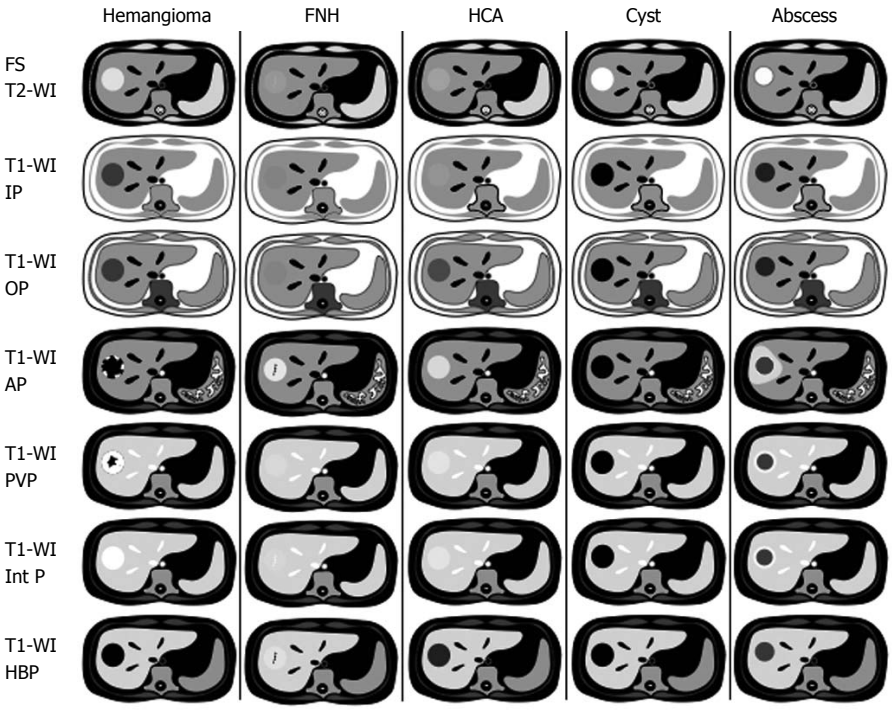

Figure 10 Stereotypical simplified schema, showing magnetic resonance imaging features of benign focal liver lesions. FNH: Focal nodular hyperplasia; HCA: Hepatocellular adenoma; FS T2-WI: Fat-suppressed T2-weighted image; T1-WI IP: T1-weighted in-phase image; T1-WI OP: T1-weighted out-of-phase image; T1-WI AP: Post-contrast fat-suppressed T1-weighted image at the late arterial phase; T1-WI PVP: Post-contrast fat-suppressed T1-weighted image at the portal-venous phase; T1-WI Inter P: Post-contrast fat-suppressed T1-weighted image at the interstitial phase; T1-WI HBP: Post-contrast fat-suppressed T1-weighted image at the hepatobiliary phase (with hepatocyte-specific contrast agent).

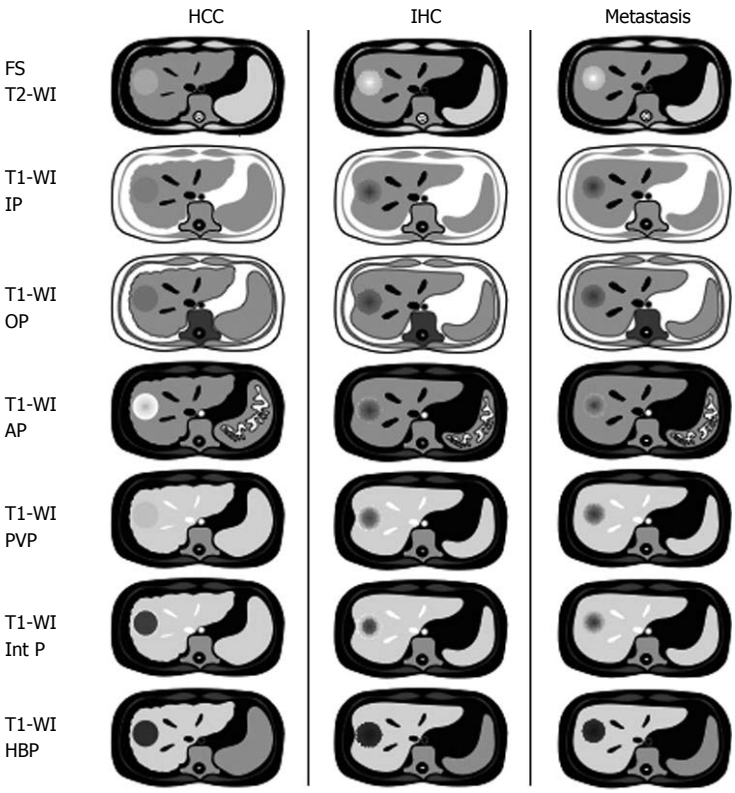

Figure 11 Stereotypical simplified schema, showing magnetic resonance imaging features of malignant focal liver lesions. The represented metastasis exemplifies the typical appearance of a hypovascular metastasis (the most common type). FS T2-WI: Fat-suppressed T2-weighted image; T1-WI IP: T1-weighted in-phase image; T1-WI OP: T1-weighted out-of-phase image; T1-WI AP: Post-contrast fat-suppressed T1-weighted image at the late arterial phase; T1-WI PVP: Post-contrast fat-suppressed T1-weighted image at the portal-venous phase; T1-WI Inter P: Post-contrast fat-suppressed T1-weighted image at the interstitial phase; T1-WI HBP: Post-contrast fat-suppressed T1-weighted image at the hepatobiliary phase (with hepatocyte-specific contrast agent); HCC: Hepatocellular carcinomas; IHC: Intrahepatic cholangiocarcinomas.

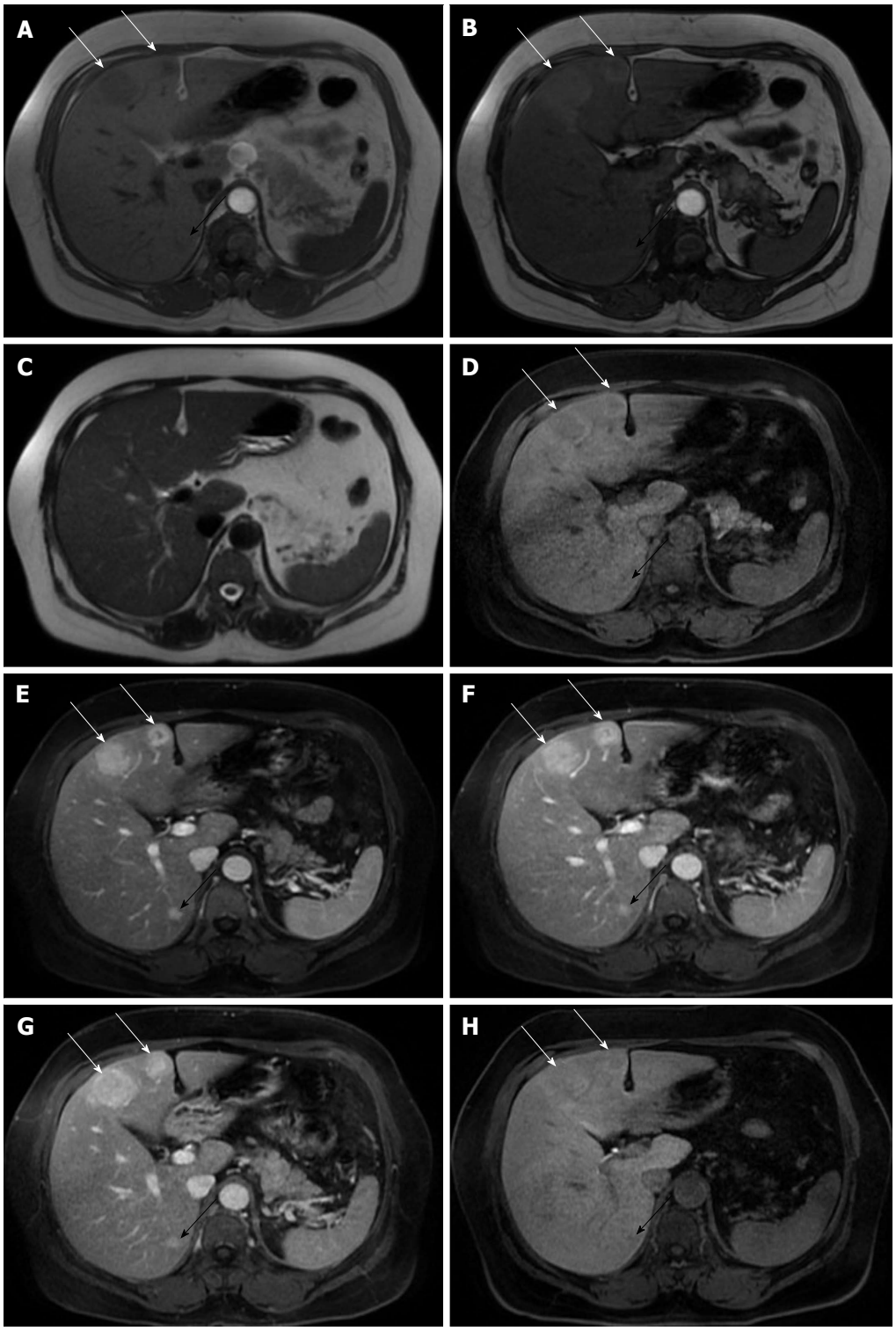

Figure 12 Multiple focal nodular hyperplasias. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and post hepatocyte-specific contrast agent (Eovist®) fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F), interstitial (G) and hepatobiliary (H) phases. There are two focal nodular hyperplasias on the left lobe (white arrows) and one small FNH on the right lobe (black arrow). The liver parenchyma shows drop of signal in the opposed-phase (B) comparing to the in-phase images (A), indicating moderate parenchymal fat deposition. Note that the lesions do not show drop in signal in the opposed-phase (B). All lesions are isointense comparing to the surrounding liver on T2-WI (C), showing uniform blush on the early post-contrast images (E). In this case the lesions enhancement do not fade to isointensity on the delayed post-contrast images (F and G) due to the presence of moderate fat deposition in the liver parenchyma. On the hepatobiliary phase, 20 min after the administration the hepatocyte-specific contrast agent, the lesions show uptake of the contrast agent. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

be assessed by the unenhanced sequences. The signal intensity of these lesions is similar to the liver parenchyma. The multi-phasic dynamic post-contrast imaging further helps in characterization, while HSA may differentiate between normal or abnormal hepatocyte function.

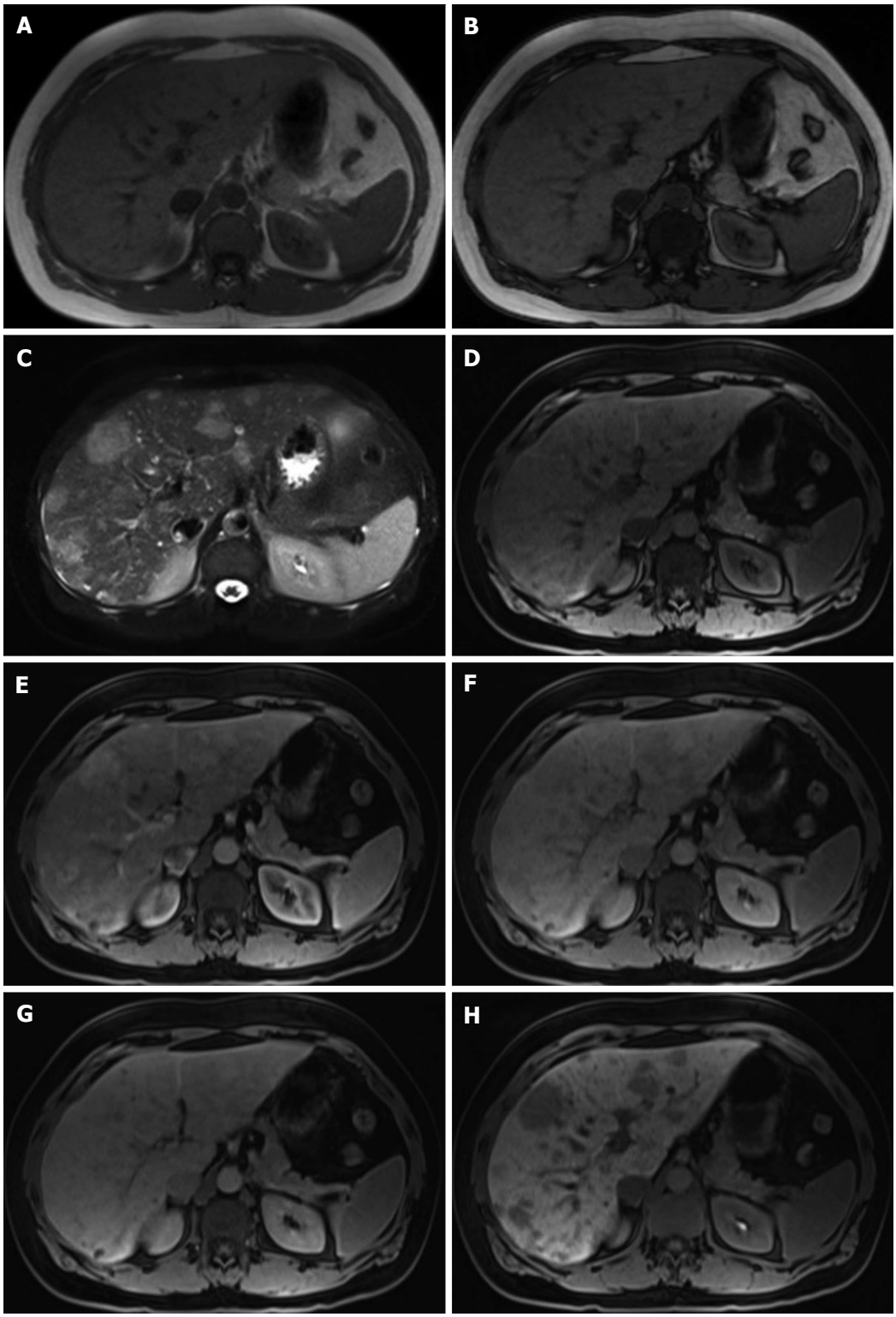

Figure 13 Adenomatosis. In- (A) and opposed-phase (B) GRE T1-WI, fat-suppressed FSE T2-WI (C), pre (D) and post hepatocyte-specific contrast agent (Eovist®) fat-suppressed 3D-GRE T1-WI at the arterial (E), portal venous (F), interstitial (G) and hepatobiliary (H) phases. There are multiple lesions (> 10 in number) scattered throughout the hepatic parenchyma, barely visible on unenhanced T1-WI (A, B and D) and hyperintense on T2-WI (C). The lesions show arterial enhancement (E), which fades to almost isointensity on the delayed post-contrast images (F and G). On the hepatobiliary phase, acquired 20 min after the hepatocyte-specific contrast agent, all lesions appear with low signal intensity comparing to the surrounding liver. GRE: Gradient-echo; FSE: Fast spinecho; T1-WI: T1-weighted images.

Although prone to restraint, an exercise of a simplistic approach taking into consideration the clinical information and the MR findings can be performed. In young women, a nodular liver lesion that looks similar in signal to the remaining parenchyma on unenhanced MR (suggesting hepatocellular origin), and showing strong enhancement on the late arterial phase, the diagnosis of FNH, or less likely, HCA should be considered. MRI

with HSA usually allows the distinction between these two lesions.

Hemangiomas usually display pathognomonic imaging features, namely the moderately high signal intensity on T2-WI and the peripheral globular discontinuous enhancement and retention of contrast on delayed images. Furthermore, in a patient without underlying liver disease or history of extra-hepatic cancer, a lesion with moderately high signal intensity on T2-WI that strongly enhances at the arterial phase and remains high in signal intensity on subsequent phases is also typical of capillary liver hemangioma. As mentioned above, while using Eovist®, one should remind that hemangiomas can show "pseudowashout", simulating malignant lesions.

In patients with multiple liver tumors, different types of lesions frequently occur in particular combinations. The most frequent combination is hemangioma and FNH, which occurs in near 25% of patients. Less frequently, FNH and hepatocellular adenoma may present as multiple lesions and typically show similar characteristics of singular lesions (Figure 12). Liver adenomatosis (> 10 adenomas), is an uncommon entity that occurs most often in young women and has three MRI patterns that are associated with three pathologic forms as described above[101] (Figure 13).

In patients with underlying liver disease regenerative hepatocellular nodules, dysplastic nodules, and HCCs are by far the most common lesions. In this setting, a nodular liver lesion that looks similar in signal to the liver parenchyma on unenhanced MR (hepatocellular origin), and shows enhancement similar to the background liver parenchyma throughout all phases are regenerative or low-grade dysplastic nodules; if they show hyper-enhancement but no washout, they are regarded as high-grade dysplastic nodules; and if they show hyper-enhancement and delayed washout, then the diagnosis of HCC is established. Ancillary findings of mildly high T2 signal intensity or restriction on diffusion in case of hypervascular nodules, despite the presence of washout, is also very suspicious for HCC.

Although the prevalence of benign lesions in patients with cancer remains high, one should always consider the possibility of liver metastases, especially when liver lesions are small and cannot be fully characterized by other imaging methods such as CT. Multiple solid liver lesions that do not show pathognomonic appearance of any of the common benign liver lesion in a patient with a known history of extra-hepatic malignancy are very suspicious for liver metastases.

CONCLUSION

Different imaging sets can be obtained in a single MRI examination, reflecting a greater range of chemical and physical properties of both normal and abnormal tissue. MRI is capable of providing comprehensive and highly accurate diagnostic information, with the additional advantage of lack of harmful ionizing radiation. These properties make MRI the mainstay for the noninvasive evaluation of focal liver lesions. Like with other radiologic exams, the interpretation of a liver MR should be done in a by-patient fashion. The expertise of an experienced subspecialized abdominal MR radiologist is paramount to establish and maintain high-quality liver MR protocols, determine the appropriate indications for the utilization of hepatocyte *vs* extracellular contrast agents, and interpret MR studies; therefore, consistently yielding a correct diagnosis and ultimately setting the right path and pace for patients' management.

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REVIEW

Transarterial chemoembolization: Evidences from the literature and applications in hepatocellular carcinoma patients

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Abstract

Transarterial chemoembolization (TACE) is the current standard of care for patients with large or multinodular hepatocellular carcinoma (HCC), preserved liver function, absence of cancer-related symptoms and no evidence of vascular invasion or extrahepatic spread (*i.e.*, those classified as intermediate stage according to the Barcelona Clinic Liver Cancer staging system). The rationale for TACE is that the intra-arterial injection of a chemotherapeutic drug such as doxorubicin or cisplatin followed by embolization of the blood vessel will result in a strong cytotoxic effect enhanced by ischemia. However, TACE is a very heterogeneous operative technique and varies in terms of chemotherapeutic agents, treatment devices and schedule. In order to overcome the major drawbacks of conventional TACE (cTACE), non-resorbable drug-eluting beads (DEBs) loaded with cytotoxic drugs have been developed. DEBs are able to slowly release the drug upon injection and increase the intensity and duration of ischemia while enhancing the drug delivery to the tumor. Unfortunately, despite the theoretical advantages of this new device and the promising results of the pivotal studies, definitive data in favor of its superiority over cTACE are still lacking. The recommendation for TACE as the standard-of-care for intermediate-stage HCC is based on the demonstration of improved survival compared with best supportive care or suboptimal therapies in a meta-analysis of six randomized controlled trials, but other therapeutic options (namely, surgery and radioembolization) proved competitive in selected subsets of intermediate HCC patients. Other potential fields of application of TACE in hepato-oncology are the pre-transplant setting (as downstaging/bridging treatment) and the early stage (in patients unsuitable to curative therapy). The potential of TACE in selected

advanced patients with segmental portal vein thrombosis and preserved liver function deserves further reports.

Key words: Transarterial chemoembolization; Loco-regional treatment; Hepatocellular carcinoma; Liver cancer; Hepatocarcinoma; Radiofrequency ablation

Core tip: Transarterial chemoembolization (TACE) represents the standard of care for patients with large or multinodular hepatocellular carcinoma (HCC). However, TACE is a heterogeneous technique varying in terms of chemotherapeutic agents, devices and schedule. In order to overcome these drawbacks of conventional TACE (cTACE), drug-eluting beads have been developed. Unfortunately, despite its theoretical advantages, definitive data in favor of its superiority over cTACE are still lacking. TACE represents the standard-of-care for intermediate-stage HCC, in competition with other therapeutic options (surgery and radioembolization). Other fields of application are the pre-transplant setting and the early stage (in patients unsuitable to curative therapy).

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INTRODUCTION

Transarterial chemoembolization (TACE) is the current standard of care for patients with large or multinodular hepatocellular carcinoma (HCC), preserved liver function, absence of cancer-related symptoms, and no evidence of vascular invasion or extrahepatic spread [*i.e.*, those classified as intermediate stage according to the Barcelona Clinic Liver Cancer (BCLC) staging system][1,2]. Furthermore, in clinical practice, many patients in the early stage (*i.e.*, single nodule or up to 3 nodules under 3 cm) carrying contraindications to curative approaches - liver resection, liver transplantation (LT) or radiofrequency ablation (RFA) - are treated with TACE.

The rationale for TACE is that the intra-arterial injection of a chemotherapeutic drug such as doxorubicin or cisplatin followed by embolization of the blood vessel will result in a strong cytotoxic effect enhanced by ischemia[3]. The embolization end point is usually defined as stasis in the second- or third-order branches of the lobar hepatic artery and injection should be continued until near stasis is observed in the artery directly feeding the tumor (*i.e.*, the contrast column should clear within 2-5 heartbeats)[4].

TACE is a very heterogeneous operative technique and varies in terms of chemotherapeutic agents, treatment devices and schedule. Such heterogeneity explains the great range in terms of efficacy outcomes: a recent systematic review reported mean overall survival (OS) times of 3.422 up to more than 40 mo, with a median of 16.5 mo[5]. The best outcomes in terms of OS reported so far are 48 mo in a series published by the Barcelona group[6].

INDICATIONS

Patients should present a relatively well preserved liver function, defined as Child-Pugh (CP) $\leqslant$ B7 stage without ascites according to European Association for the Study of the Liver (EASL) guidelines[2] or only CP A according to the more conservative American Association for the Study of Liver Diseases (AASLD) guidelines[1].

Absolute contraindications to TACE are generally related to decompensated cirrhosis or impaired portal blood flow[1,2]. Other absolute contraindication, supported by the expert opinion, is represented by extensive tumors massively replacing both entire lobes, whereas a tumor size $\geqslant$ 10 cm, the bile-duct occlusion and untreated varices at high risk of bleeding constitute relative contraindication rather than absolute ones[5]. Main absolute and relative contraindications to TACE are reported in Table 1.

Although the adverse events associated with TACE are generally transient and easily manageable, they are very common with 35%[7] to 100%[8] of treated patients experiencing post-embolization syndrome (defined by the occurrence of abdominal pain, fever and nausea). Treatment-related deaths are expected in less than 2% of cases if proper selection of candidates is in place[9].

Therefore, TACE appears as a safe treatment in selected candidates, as defined by current guidelines.

TREATMENT SCHEDULE

Current evidence suggests that one cycle of TACE may not be sufficient for effective treatment of intermediate-stage HCC. On the other hand, there is evidence suggesting that repeating TACE prolongs survival; however, current guidelines do not specify the criteria for treatment repetition. In particular, it should be noted that in bilobar tumors, the two hepatic lobes usually have to be treated in separate treatment sessions 2-4 wk apart.

There are no solid data to suggest that "on-demand" TACE (*i.e.*, number of sessions on the basis of tumor response after each TACE cycle) is more or less effective than scheduled TACE (pre-defined number of sessions regardless of "at interim" response or safety evaluations) for improving patient survival. In fact, although scheduled strategy is more concordant with the general principle of oncologic therapy, which uses standard chemotherapeutic sessions based on the

Table 1 Absolute and relative contraindications to transarterial chemoembolization

Absolute contraindications
Decompensated cirrhosis (Child-Pugh ⩾ B8)
Extensive tumor with massive replacement of both entire lobes
Severely reduced portal vein flow
Technical impediments to hepatic intra-arterial treatment
Relative contraindications
Kidney failure
Severe cardiopulmonary comorbidities
Tumor size ⩾ 10 cm
Untreated varices at high risk of bleeding
Bile-duct occlusion

cell cycle, however, there is evidence suggesting that the repetition of TACE with an aggressive schedule increases the incidence of adverse events[10]. Therefore, the experts in the field propose the on-demand repetition with longer intervals between treatments, rather than a regular predefined schedule[5,11]. This has been recently confirmed by Terzi *et al*[12] in a series of 151 patients treated with on-demand conventional TACE (cTACE). In their analysis, a second TACE course was administered to 65% of patients who experienced a recurrence after the complete response and to only 41% of patients non responder to the first course. Therefore, the results of this study demonstrate that only approximately half of the patients with incomplete response or recurrences were eligible for repeated TACE, mainly because of tumor burden growth and liver function impairment[12]. These findings stand for an on-demand strategy to be "tailored" according to individual patients' characteristics.

REPEATED TACE: IS IT POSSIBLE A SCORE FOR ALL SEASONS?

What remains to be definitively established is the maximum number of repeated TACE procedures that should be administered before switching to another therapeutic option or stopping treatment. Applying TACE procedures up to 3 to 4 times per year[11] and switching in absence of response to at least 2 sessions[5] has been recommended in absence of definitive evidence of an optimal retreatment strategy because more intensive regimens might induce liver failure in an unacceptable proportion of patients. A review of cohort and randomized controlled trials (RCTs) reported a mean number of TACE courses of 2.5 ± 1.5 per patient[13], but in the common clinical practice an even greater number of repeated sessions is undertaken.

To help the hepatologists to select appropriate candidates for starting or repeating TACE, several prognostic indices were introduced in the past, but none of them were universally accepted since they resulted difficult to implement or insufficiently discriminatory[14,15]. More recently, a number of other scores and nomograms have been proposed, particularly: the hepatoma arterial-embolization prognostic score published by Kadalayil *et al*[16] in 2013, based on albumin, bilirubin, alpha-fetoprotein (AFP) and tumour size; the assessment for retreatment with TACE (ART) score proposed by Sieghart *et al*[17] in 2013, considering aspartate transaminase and CP increase after the first session together with tumor response; the ABCR score published by Adhoute *et al*[18] in 2014 on the basis of AFP and BCLC stage at baseline together with CP increase and tumor response after TACE; the inflammation based index score, that combines C-reactive protein and serum albumin, proposed by Pinato *et al*[19] and applied to TACE patients in 2015. Other proposed scores and nomograms are reported in Table 2[20-22].

Unfortunately, none of these new prognostic systems have been unequivocally confirmed in clinical practice[23-26]. In fact, all these efforts, although properly conducted, suffer from overfitting: a phenomenon occurring when a model maximizes its performance on some set of data but its predictive performance is not confirmed elsewhere due to random fluctuations of patients' characteristics in different clinical and demographical backgrounds. The very fact that so different scores keep on being proposed confirms and gives proof of this concept. When a model is built, as in the case of the aforementioned studies, the score is tested in a different but "plausibly related" cohort and that is called external validation; unfortunately, external validation has been found to show sufficient power to detect clinically important changes in performance only when substantial sample sizes are available, that is not common in clinical research[27]. With smaller series, as in the case of most of the above reported papers, the sole external validation may lead to an overestimation of the performance of the model. In attendance of larger multicenter series and more reliable statistical tools (for instance bootstrap sampling or internal validation)[28], an unequivocally accepted prognostic system able to guide the decision of TACE repetition remains an unmet need. The detailed list of the proposed scoring systems for HCC patients undergoing TACE is reported in Table 2.

USEFULNESS OF DRUG INJECTION

Robust data in favor of a clear superiority of conventional TACE over transarterial embolization (TAE) are lacking[29]. A RCT comparing cTACE, TAE and best supportive care (BSC) was prematurely terminated due to the superiority of cTACE over BSC (see below)[30]. Unfortunately, this prevented the possibility to verify the efficacy of TAE, which could be hypothesized based on the trend observed in OS[30]. Similarly, no difference in terms of survival rates was reported between cisplatin-based TACE and TAE in a small Chinese RCT[31]. On the other hand, the added value of the chemotherapeutic agent (doxorubicin) in drug-eluting bead (DEB)-TACE over bland TAE has been recently demonstrated in a Greek RCT, which found an increase in time to progression (TTP) from 36.2 ± 9 wk up to 42.4 ± 9.5 wk (P = 0.008) in

Table 2 Proposed scoring systems for hepatocellular carcinoma patients undergoing transarterial chemoembolization

Ref.	Variables considered	Aim
Lladó *et al*[15]	AFP (> 400 UI/L), tumor size (> 50%) and CP score	Treatment selection
Kadalayil *et al*[16]	Albumin < 3.6 g/L, bilirubin > 17 μmol/L, AFP > 400 ng/mL and dominant tumor size > 7 cm	Treatment selection
Sieghart *et al*[17]	Increase of AST by > 25% and of CP score from baseline, tumor response	Treatment repetition
Adhoute *et al*[18]	BCLC, AFP (> 200 ng/mL), increase in CP score by ≥ 2 from baseline and tumor response	Treatment repetition
Pinato *et al*[19]	Normalization of CRP and serum albumin after TACE	Treatment repetition
Hucke *et al*[20]	Albumin level, tumour burden (reference: up-to-7 criteria) and CRP(≥ 1 mg/dL)	Treatment selection
Xu *et al*[21]	PVT, tumor number, tumor capsule, AFP, AST and ICR	Treatment selection
Sciarra *et al*[22]	CD34 and VEGF staining[1]	Treatment selection

[1]Assessed in tumor biopsy. AFP: Alpha-fetoprotein; CP: Child-Pugh; AST: Aspartate transaminase; BCLC: Barcelona Clinic Liver Cancer; CRP: C-reactive protein; TACE: Transarterial chemoembolization; PVT: Portal vein thrombosis; ICR: Indocyanin retention test; VEGF: Vascular endothelial growth factor.

DEB-TACE patients[32]. Another investigation assessed the degree of necrosis in explanted livers after epirubicin DEB-TACE versus TAE and found tripled complete necrosis rates (77% *vs* 27% of lesions) in the DEB-TACE group[33].

There is no consensus on the optimal chemotherapeutic agent to use in TACE. Worldwide, the most popular anticancer drug injected is doxorubicin. In cTACE, the dose of doxorubicin typically ranges from 30 to 75 mg/m^2 (to a maximum of 150 mg) mixed with 5 to 20 mL of lipiodol, followed by mechanical embolization with an embolic agent, as Gelfoam[4]. In DEB-TACE, the planned dose of doxorubicin should depend on the extent of the liver tumor burden: as a general rule, for disease within the Milan criteria each single treatment should include a planned dose of up to 75 mg doxorubicin loaded into one vial of DC Bead, whereas for disease beyond the Milan criteria, the dose should be of up to 150 mg loaded into two vials of DC Bead[4].

DEB-TACE *VS* CTACE

Ideally, the injected chemotherapeutic should be retained in the tumor and be gradually released to avoid systemic toxicity. However, even if suspended in lipiodol as in the case of cTACE, its selective injection is associated to significant passage into the systemic circulation. Other important limitation of conventional TACE has been the lack of standardization of the technique. In fact, the emulsification of the drug and lipiodol is prepared extemporaneously and hence is operator-dependent (not standardized) and is unstable. Therefore, to overcome the major drawbacks of cTACE, non-resorbable embolic microspheres loaded with cytotoxic drugs (DEBs) have been developed. In fact, DEBs are able to slowly release the drug upon injection and increase the intensity and duration of ischemia while enhancing the drug delivery to the tumor[4].

The first report on the efficacy of DEB-TACE was the phase Ⅱ study by Varela *et al*[34]. In this pivotal paper, 27 CP A HCC patients received two DEB-TACE (500-700 μm particles) sessions at 2-mo intervals: objective response rate was 66.6% (whereof 26% were complete responses). Serial blood samples were obtained in 13 patients to determine doxorubicin maximal concentration and area under the curve, which resulted significantly lower in DEB-TACE patients as compared to an historical cohort of cTACE patients (P = 0.00002 and P = 0.001, respectively). Furthermore, DEB-TACE was well tolerated with only two cases of severe adverse events (namely, liver abscesses)[34]. These results were confirmed by Poon *et al*[35], who used the highest dose possible of doxorubicin (150 mg). In both studies, none of treated patients presented doxorubicin-related systemic toxicity (alopecia, bone marrow toxicity, dyspnea or pulmonary embolism)[34,35].

In light of successive clinical and in-animal studies[36,37], use of 100-300 μm beads is actually recommended, based on the demonstration that such small particles are delivered inside the tumor or in close proximity to the tumor margins and thus are ideal for drug delivery or precise embolization[4].

Despite the promising results of these preliminary studies and the aforementioned theoretical advantages of DEB-TACE, a clear superiority of one technique over the other is still lacking.

The comparison between cTACE and DEBs has been object of 12 studies (whereof 4 RCTs)[38-49] and 3 recent meta-analyses[50-52] (Table 3). In the most recent meta-analysis, a significantly better objective tumor response rate was found for DEB-TACE than for conventional TACE [odds ratio (OR) = 1.84, 95%CI: 1.02-3.33; P = 0.04], but Mantel-Haenzel OR for 3-year survival (reported in 4 studies) was non significant (0.77, CI: 0.55-1.06, P = 0.11)[50]. With regard to toxicity, either overall and severe adverse events were similar in both groups, with post-embolization syndrome occurring most commonly[50,51].

Although a clear superiority of DEB-TACE is still lacking, new micro-particles have been recently introduced in the clinical practice. As previously mentioned, small diameter beads have been shown to inflict pan-necrosis of the target lesion since smaller bead diameters achieve a more distal embolization, thus also obstructing collateral channels[35-37]. Therefore, smaller particles have been recently tested with promising results[53-55], but broader cohort studies and RCTs are warranted to validate such findings.

Table 3 Studies comparing conventional and drug-eluting beads transarterial chemoembolization in hepatocellular carcinoma patients

Ref.	Arm	Drug	Sample size	Study design	Region
[1]Nicolini *et al*[38]	DEB-TACE	Doxorubicin	22	R	Italy
	cTACE	Epirubicin	16		
[1]Frenette *et al*[39]	DEB-TACE	Doxorubicin	35	R	United States
	cTACE	Doxorubicin	76		
Song *et al*[40]	DEB-TACE	Doxorubicin	60	R	South Korea
	cTACE	Doxorubicin or Epirubicin/Cisplatin	69		
Sacco *et al*[41]	DEB-TACE	Doxorubicin	33	RCT	Italy
	cTACE	Doxorubicin	34		
van Malenstein *et al*[42]	DEB-TACE	Doxorubicin	16	RCT	Belgium
	cTACE	Doxorubicin	14		
Lammer *et al*[43]	DEB-TACE	Doxorubicin	93	RCT	Europe
	cTACE	Doxorubicin	108		
Golfieri *et al*[44]	DEB-TACE	Doxorubicin	89	RCT	Italy
	cTACE	Epirubicin	88		
Ferrer Puchol *et al*[45]	DEB-TACE	Doxorubicin	47	P	Spain
	cTACE	Doxorubicin	25		
Dhanasekaran *et al*[46]	DEB-TACE	Doxorubicin	45	R	United States
	cTACE	Doxorubicin/Cisplatin/Mytomicin-C	26		
Wiggermann *et al*[47]	DEB-TACE	Epirubicin	22	R	Germany
	cTACE	Cisplatin	22		
Recchia *et al*[48]	DEB-TACE	Doxorubicin	35	P	Italy
	cTACE	Doxorubicin	70		
Megìas Vericat *et al*[49]	DEB-TACE	Doxorubicin	30	R	Spain
	cTACE	DOxorubicin	30		

[1]Study conducted on transplanted patients. DEB-TACE: Drug-eluting beads transarterial chemoembolization; cTACE: Conventional transarterial chemoembolization; R: Retrospective; RCT: Randomizes controlled trial; P: Prospective.

APPLICATIONS OF TACE IN HEPATO-ONCOLOGY

Intermediate stage

The recommendation for TACE as the standard-of-care for intermediate-stage HCC is based on the demonstration of improved survival compared with best supportive care or suboptimal therapies in a meta-analysis of six RCTs[56]. However, there was considerable heterogeneity between the individual study designs (including patient populations and TACE technique) as well as the study results, with only two[30,57] of the six individual studies that reported 2-year survival rates showing a statistically significant improvement compared with conservative management (relative risk of death after 2 years: 0.53, $P = 0.017$). Results from other two meta-analyses confirmed that TACE improved survival outcomes compared with conservative management, however, both meta-analyses also concluded that there were other treatment options (such as TAE or ethanol injection) as effective as, if not superior to, TACE for the treatment of unresectable HCC[58,59]. Furthermore, intermediate-stage HCC includes a heterogeneous population of patients varying widely in terms of tumour burden, liver function and disease etiology[11]. In fact, it should be noted that the previously mentioned studies included patients with HCC described as "unresectable" rather than those with HCC classified as intermediate according to the BCLC schema.

Overall, the expected survival for untreated intermediate HCC is 16 mo, whereas after TACE increased up to 20 in the first studies[56]. However, these studies compared TACE to BSC and not to other treatment modalities such as surgery. Several reports on expanding criteria for resection in HCC have been published in the last years. In fact, two retrospective studies[60,61] and, above all, a RCT[62] explored the comparative effectiveness of surgery (partial hepatectomy) with respect to cTACE for intermediate patients. In the Chinese RCT, median survival was 41 mo (range 1-50 mo) after surgery *vs* only 14 mo (range 5-47 mo) after TACE ($P < 0.001$). However, it should be noticed that in both study groups, median tumor size was beyond 7 cm, a value representing a suboptimal indication to TACE[62]. This may explain the relatively poor outcomes observed in TACE patients, that resulted very far from the most recent studies in the field[6,63].

On the other hand, besides the attempt to expand criteria for radical treatments, also the recently developed new loco-regional techniques have challenged the assumption of TACE as standard of care for BCLC B patients. Transarterial radioembolization (TARE) with yttrium 90 has gained increasing attention for intermediate and advanced patients in the last years[64-66]. Salem *et al*[67] retrospectively compared data from 245 patients (122 who received chemoembolization and 123 who received radioembolization) and reported longer TTP following radioembolization than chemoembolization (13.3 mo *vs* 9.4 mo, $P = 0.047$) but similar median OS (17.5 mo *vs* 17.2 mo, $P = 0.42$) in BCLC B patients. Therefore, in this landmark paper by the Chicago group, TARE resulted in longer time-to-progression and less

toxicity than chemoembolization[67]. Post-hoc analyses of sample size indicated that a randomized study with > 1000 patients would be required to establish equivalence of survival times between patients given the different therapies, a cohort not easy to collect in the clinical practice[67,68]. Other retrospective reports and a small RCTs confirmed the non significant superiority of one technique over the other[69-71].

In conclusion, in absence of further solid data provided by large RCTs, TACE remains the standard of care for intermediate HCC patients, with surgery and TARE as competitive options in case of compensated cirrhosis (CP A) or more advanced tumor burden, respectively.

Early stage

The EASL and AASLD guidelines recommend that the first option for HCC patients within Milan criteria should be hepatic resection or LT[1,2]. Nevertheless, some patients may be poor surgical candidates and the alternative is a variety of loco-regional ablation techniques. Of these, RFA is considered the treatment of choice for these patients, recently reported to be as effective for small HCCs (BCLC 0) as surgical resection[72-74]. However, some tumors with a subcapsular or dome location and tumors adjacent to intestinal loops or the main bile duct may be unsuitable for RFA and in such cases TACE can be used as therapy. Recently, Hsu *et al*[75] investigated the clinical outcomes of Milan-in HCC patients undergoing RFA (n = 315) or cTACE (n = 215). In the univariate survival analysis, the RFA group had a significantly better long-term survival than the TACE group (the 1-, 3-, and 5-year survival rates were 93%, 89%, and 72% for RFA, and 63%, 55%, and 43 % for TACE, P = 0.048), but after propensity-score matching (selecting 101 patients from each treatment arm) such a difference was lost (1-, 3-, and 5-year survival rates were 85%, 60%, and 41% for RFA, and 86%, 55%, and 36% for TACE; P = 0.476)[75]. However, patients undergoing TACE had a significantly higher cumulative recurrence rate than patients undergoing RFA (P = 0.023), hence, this study indicates that TACE and RFA lead to comparable long-term survival but differ in recurrence rate for HCC patients within the Milan criteria[75]. In subgroup analysis, patients with a smaller total tumor volume (< 11 cm^3, equivalent to a single nodule 2.8 cm in diameter) were found likely to benefit more from RFA with respect to TACE[75]. A probable reason for these results is that RFA has a less satisfactory effect on medium tumors (3.1-5 cm in diameter) and multiple tumors[76-78].

Following the conclusions of this paper, Kim *et al*[79] have recently compared the two treatments in 287 very early (BCLC 0) HCC patients (122 and 165 patients treated with cTACE and RFA, respectively). In this study, RFA and TACE did not differ significantly in terms of mean survival (80.0 ± 2.3 mo and 72.1 ± 3.2 mo, respectively; P = 0.079), but objective response rate (100% and 95.9% in the RFA and TACE group, respectively; P = 0.013) and median TTP were significantly in favor of RFA (27.0 ± 3.8 mo after RFA and 18.0 ± 2.9 mo after TACE; P = 0.034)[79]. Therefore, although the study by Kim *et al*[79] does not strongly support the superiority of RFA over TACE as no statistically significant difference was noted in terms of OS, however, RFA led to better tumor responses and was associated with delayed tumor progression compared with TACE.

The aforementioned study suggests RFA as first-line treatment for unresectable early/very early HCC patients, whereas TACE may be considered a viable alternative when RFA is not feasible.

Downstaging/bridging

TACE is the most used treatment for patients in waiting list for LT[80].

The aims of bridging treatments include decreasing the waiting list dropout rate before transplantation, reducing HCC recurrence after LT and improving post-transplant overall survival.

TACE has been extensively used in the past as a bridging treatment to LT and a number of studies have shown that it is an effective therapy in terms of adequate tumor necrosis achievement at explant analysis with complete tumor necrosis rates ranging between 27% and 57% in patients within Milan criteria[81,82].

These results are certainly of interest, considering that RFA leads to superior complete necrosis rates (between 50% and 78%) in single HCCs up to 3 cm, but significantly poorer outcomes in larger or multiple neoplasms (necrosis rate between 13% and 43%)[83-85].

The effectiveness of TARE has recently been evaluated by Riaz *et al*[86], who studied 38 nodules in 35 patients treated with radioembolization before LT. In this study, at explant analysis, 23 of the 38 target lesions (61%) showed complete tumor necrosis; in particular, complete tumor ablation was detected in 89%, 65%, and 33% of lesions smaller than 3 cm, between 3 and 5 cm, and larger than 5 cm, respectively[86]. The same Group retrospectively compared effectiveness of TACE and TARE in T3 HCC patients (*i.e.*, beyond conventional criteria): down-staging rate was 58% after TARE *vs* 31% after TACE (P < 0.05)[87].

In conclusion, no definitive recommendation can be made for one type of loco-regional therapy over others in the pre-transplant setting. However, on the basis of the aforementioned studies, RFA could be considered as the first-line treatment for single lesions up to 3 cm, in which complete tumor necrosis has been shown in more than 50% of cases at explant analysis[83-85]. TACE should be preferred for treating lesions > 3 cm because its effectiveness appears to be better in well-vascularized tumors with large feeding arteries.

Advanced stage

Advanced HCC (*i.e.*, BCLC stage C) is characterized by an Eastern Cooperative Oncology Group performance status of 1-2 and/or the presence of portal vein thrombosis (PVT) or extrahepatic metastases. According

to current guidelines, advanced HCC patients can only receive sorafenib while it is generally accepted that TACE is not recommended in cases of macroscopic portal vein invasion because of the potentially increased risk of liver failure[1,2]. Recently, however, some prospective controlled trials have shown the survival benefit of TACE over BSC in advanced HCC patients with PVT[88,89]. Therefore, the clear effects and safety of TACE in these patients remain controversial. A recent meta-analysis of 8 studies (whereof 3 prospective) has summarized the published results on this regard: TACE resulted potentially suitable and safe for advanced HCC patients with PVT with a low rate of fatal complications[90]. Furthermore, for selected patients (those with established collateral circulation and good liver function), TACE treatment prolonged survival[90]. However, the results of this meta-analysis should be interpreted with caution because all the included studies were conducted in Asia (hence, it is uncertain the applicability of these findings to Western settings) and patients with better liver function tended to be selected into the TACE group, whereas those decompensated tended to be treated with BSC. Moreover, sorafenib, and not BSC, is the reference standard treatment for advanced-stage HCC, hence, direct comparisons between the two therapies are needed.

The only head-to-head comparison between the two treatments published so far, is a retrospective European study delivered by the Vienna group[91]. By the way, even in this well written paper, an underlying selection bias can be detected, as thrombosis of the main trunk of portal vein (well-known as at poorer prognosis) was more frequently present in the sorafenib group than in the TACE group (25% *vs* 3%). Median TTP was similar between the two treatment groups (P = 0.737) as well as median OS (9.2 mo, 95%CI: 6.1-12.3 mo after TACE *vs* 7.4 mo, 95%CI: 5.6-9.2 mo in patients treated with sorafenib, P = 0.377)[91]. Interestingly, in the Austrian study, TACE achieved promising outcomes (median OS of 14 mo) in selected advanced patients (CP A and segmental PVT), a result confirmed in other retrospective reports[92]. However, in the TACE group, 13 patients experienced severe adverse events and 4 treatment-related deaths, thus pointing out serious concerns on the safety of TACE in this setting[91].

Therefore, TACE might be a reasonable alternative for selected advanced patients (segmental PVT and CP A) who do not have access or are intolerant/unsuitable to sorafenib or TARE, but the particular attention to be paid to the safety profile restricts this therapeutic opportunity to highly-experienced centers.

Combined regimens

A meta-analysis of 10 randomized trials and 18 observational studies including 2497 patients showed that the combination of TACE with other treatments, such as ethanol injection, external radiotherapy and high-intensity focused ultrasound, result in better survival outcomes and similar side effects than TACE alone[93]. However, for each combination, the number of studies were mostly inadequate to provide a definitive recommendation, thus further well-organized randomized trials are needed to confirm these findings.

TACE is associated with local and systemic increase in vascular endothelial growth factor, since embolization interrupts blood supply to the tumor, inducing hypoxia and necrosis[94]. These observations suggest that an antiangiogenetic agent (namely, sorafenib) may counteract TACE-induced angiogenesis, thus improving the post-procedural outcomes[95,96]. Two important RCTs have explored the feasibility and the efficacy of the combined regimen, without finding any definitive evidence in favor of the association of sorafenib with TACE[97,98]. However, since other smaller RCTs and retrospective studies provided discordant results, combined regimens between antiangiogenetic agents and TACE remain an interesting field of research in hepato-oncology[99-102].

CONCLUSION

TACE covers a broad spectrum of therapeutic indications in hepato-oncology and, if the proper selection of candidates is followed, represents a safe and effective treatment. Further studies are needed to correctly expand treatment indications and define the more appropriate combined regimens with other loco-regional therapies or systemic drugs.

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REVIEW

Biological features and biomarkers in hepatocellular carcinoma

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Abstract

Similar to other cancers, a multistep process of carcinogenesis is observed in hepatocellular carcinoma (HCC). Although the mechanisms underlying the development of HCC have been investigated in terms of oncology, virology, and stem cell biology, the whole picture of hepatocarcinogenesis remains to be elucidated. Recent progress in molecular biology has provided clues to the underlying cause of various diseases. In particular, sequencing technologies, such as whole genome and exome sequencing analyses, have made an impact on genomic research on a variety of cancers including HCC. Comprehensive genomic analyses have detected numerous abnormal genetic alterations, such as mutations and copy number alterations. Based on these findings, signaling pathways and cancer-related genes involved in hepatocarcinogenesis could be analyzed in detail. Simultaneously, a number of novel biomarkers, both from tissue and blood samples, have been recently reported. These biomarkers have been successfully applied to early diagnosis and prognostic prediction of patients with HCC. In this review, we focus on the recent developments in molecular cancer research on HCC and explain the biological features and novel biomarkers.

Key words: Hepatocellular carcinoma; Heterogenity; Molecular biology; Oncology; Sorafenib

Core tip: Recent progress in molecular biology enabled understanding of the mechanisms underlying hepatocarcinogenesis and identification of useful biomarkers. According to these findings, further efforts would be needed to improve understanding of these molecular mechanisms and to establish novel therapeutic

approaches.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths, accounting for approximately 600000 deaths annually worldwide and more than 30000 deaths annually in Japan[1,2]. It is well-known that hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, and nonalcoholic fatty liver disease are the major risk factors for hepatocarcinogenesis[3]. Majority of patients with HCC are HBV or HCV carriers[4,5]. In HBV X protein (HBx) transgenic mice, HCC developed within one year after birth[6]. Similarly, HCV core transgenic mice exhibited hepatic steatosis several months after birth and eventually developed HCC[7]. These findings implicate that chronic infection with HBV and HCV have a direct action on hepatocarcinogenesis. Moreover, the incidence of HCC in patients with metabolic syndrome or nonalcoholic steatohepatitis has been increasing[8].

It is now widely considered that accumulation of genetic and/or epigenetic alterations transforms normal cells into cancer cells through a neoplastic state. This clinically well recognized process is called "stepwise carcinogenesis"[9]. The transformed cells usually acquire unique properties, such as sustained proliferative signaling, evasion growth suppressors, resistance to cell death, ability for replicative immortality, induction of angiogenesis, and activation of invasion and metastasis[10,11]. Recent progress in molecular biology and translational science enabled characterization of cancer cells and establishment of therapeutic approaches in a wide range of cancers. Sorafenib, an oral multikinase inhibitor, has been recognized as a new molecular-targeted therapy for HCC. The agent suppresses tumor growth and angiogenesis by inhibiting the RAS/RAF/MAPK signaling pathway and tyrosine kinase receptors including vascular endothelial growth factor receptor (VEGFR)[12]. However, the prognosis of patients with HCC treated with sorafenib has not been essentially satisfactory[13,14]. Therefore, further understanding of the molecular mechanisms underlying hepatocarcinogenesis and establishment of novel therapeutic approaches remain the most important challenges.

In this review, we will summarize the recent progress in molecular cancer research on HCC and explain the molecular mechanisms underlying hepatocarcinogenesis. We will also highlight the serological and pathological biomarkers of HCC for diagnosis and prognostication.

BIOLOGICAL FEATURES OF HCC

Signaling pathways and genetic alteration in HCC

It has been documented that dysregulation of several signaling pathways including p53/RB, Wnt/β-catenin, PI3K/PTEN/Akt/mTOR pathways plays an important role in the development and progression of HCC[15,16]. It is considered that the aberrant activation or inactivation of these pathways is attributable to somatic alterations, such as mutations, changes of copy numbers, and chromosomal rearrangements[17]. These results could be applied to the classification and prognostication of HCC[18]. Among them, mutation of *p53* and *β-catenin* has long been recognized as a common genetic alteration in HCC[19,20], and it is observed in approximately 30% and 20% of HCC samples, respectively.

Recent whole genome and exome sequencing analyses enabled the surveillance of the signature of genomic alteration and identification of somatically mutated genes (Figure 1). In a study on exome sequencing of 24 HCC samples, Guichard *et al*[21] demonstrated that major pathways including Wnt/β-catenin signaling, p53/cell cycle signaling, PI3K/RAS signaling, chromatin regulation, and oxidative and endoplasmic reticulum stress signaling were commonly altered by somatic mutations or homozygous gene deletions. They found recurrent alterations in four genes (*ARID1A*, *RPS6KA3*, *NFE2L2* and *IRF2*), which were not previously reported in HCC. Particularly, *ARID1A*, a chromatin remodeling gene, was shown to be frequently mutated in alcohol-related HCC. Whole-genome sequencing (WGS) of HCC samples has also revealed recurrent somatic mutations in several genes associated with chromatin regulation, such as *ARID1A*, *ARID1B*, *ARID2*, *MLL*, *MLL3*, *BAZ2B*, *BRD8*, *BPTF*, *BRE* and *HIST1H4B*[22]. Mutations in at least one of these chromatin regulator genes were detected in more than 50% of HCC tissues. Taken together, dysregulated chromatin remodeling plays a critical role in HCC development.

Heterogenity of HCC

Genetic and functional heterogeneity in tumor-constituent cells has been observed in a wide range of cancers[23]. To explain this, a hierarchical model or cancer stem cell (CSC) model has been proposed and debated on[24]. This model postulates that a small population generates a hierarchical structure containing descendant tumor cells. However, clonal evolution model suggests that a series of clonal expansions accompanied by accumulated genetic alterations contribute to intratumor heterogeneity[25]. Recent sequencing technologies have successfully demonstrated that most tumors exhibit extensive intratumoral heterogeneity characterized by individual tumor cells showing different somatic mutation pattern[26]. Gerlinger *et al*[27] conducted exome sequencing analysis of resected renal cell carcinoma samples and demonstrated not only intratumoral heterogenity but also genetic alternations between primary

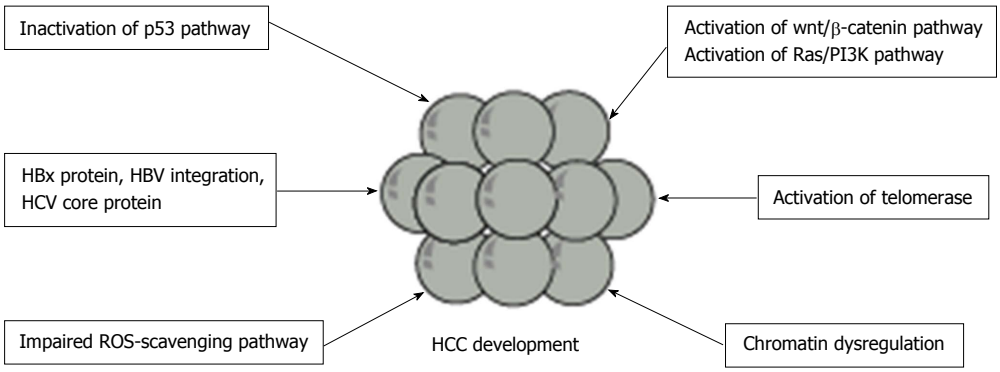

Figure 1 The major pathways responsible for the development of hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBx: HBV X protein; ROS: Reactive oxygen species.

tumor and metastatic lesions.

Multicentric tumor development is one of the most vital aspects of hepatocarcinogeneis[28]. Fujimoto *et al*[22] performed WGS on two pairs of multicentric HCV-associated HCCs and unexpectedly found out that neither common somatic mutations in coding region nor common structural alterations were observed in these tumors. These results may indicate that multicentric HCCs were derived from cells with different genetic alterations. On the other hand, Huang *et al*[29] employed exome sequencing of nine pairs of matched primary HBV-associated HCCs and portal vein tumor thrombus (PVTT) and demonstrated that more than 90% of nonsynonymous somatic mutations were shared between primary HCC and PVTT. Furthermore, 65 genes with mutations either in primary HCC or PVTT were identified. Among them, mutations in *KDM6A*, *CUL9*, *FGD6*, *AKAP3* and *RNF139* were detected in PVTT, but not in primary tumors.

HBV integration

It is well known that HBx, a multifunctional protein encoded by the HBV genome, has the ability of the transcriptional transactivation[30]. Moreover, HBx could activate the JAK/STAT signaling pathway but impair the p53 function[31,32]. Thus, HBx is deeply involved in hepatocarcinogenesis.

The integration of HBV DNA into the host genome is one of the important factors in hepatocarcinongenesis in patients with chronic HBV infection[33]. This appears to contribute to oncogene activation and/or tumor-suppressor gene inactivation. HBV integration at the Cyclin A and retinoic acid receptor β gene has been reported approximately 20 years ago[34,35]. Recently, a novel sequencing technology was successfully applied to the analyses of HBV genome integration. HBV genome integration in the telomerase reverse transcriptase (TERT) locus was observed in 4 of 11 HBV-related HCC samples examined[22]. Because activation of telomerase, encoded by the TERT, is associated with cellular immortalization, the dysregulation of TERT expression may play a crucial role in hepatocarcinogenesis. Sung *et al*[36] conducted WGS of 88 Chinese patients with HCC and noted that nearly 40% of HBV breakpoints were located around the X and core gene. Further, they revealed that in some HCC samples, five genes (*TERT*, *MLL4*, *CCNE1*, *SENP5*, and *ROCK1*) were recurrently affected by HBV integration. They reported that the number of HBV integration sites per tumor significantly correlates with serum levels of HBsAg and alfa-fetoprotein (AFP). In addition, patients with HCC with several HBV integration sites exhibited shorter survival time than those with few integration sites.

Single nucleotide polymorphisms associated with HCC development

Genome-wide association studies are microarray-oriented technologies that have been utilized to identify single nucleotide polymorphisms (SNPs) associated with many traits and diseases. Intronic SNPs in *KIF1B* and *STAT4* have been shown to be highly associated with HCC occurrence in Chinese chronic HBV carriers[37,38]. Similarly, it has been recently reported that SNPs in *MICA* and *DEPDC5* are associated with HCC development in Japanese patients with chronic HCV infection[39,40]. To confirm the utility of these SNPs as risk markers for HCC, further analyses of different populations would be needed.

Stemness features in HCC

It has been reported that signaling pathways and molecular mechanisms operating in stem cells are similar to those in cancer[41]. For example, BMI1, a polycomb gene product, is a general regulator in normal stem cell systems[42]. On the other hand, high expression levels of BMI1 were observed in various cancers. We have previously reported that fetal hepatic stem/progenitor cells transduced with BMI1 acquired enhanced self-renewal capability and tumorigenicity to generate combined HCC in a mouse transplant model[43]. Glinsky *et al*[44] analyzed gene expression profiles in wild type and BMI1$^{-/-}$ neurospheres. By comparing those to the data obtained from primary and metastatic prostate cancer, they successfully selected 11 BMI1-associated genes. This 11-gene death-from-cancer signature was validated in both epithelial and hematological malignancies.

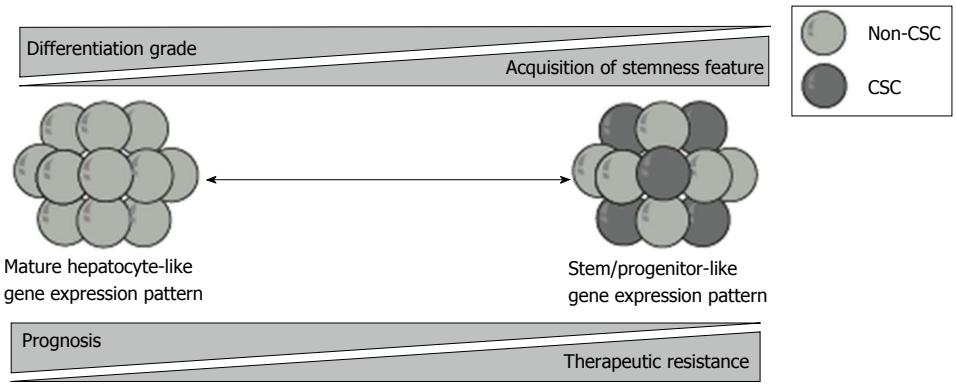

Figure 2 Cancer stem cells in hepatocellular carcinoma. Acquisition of stemness feature is closely associated with therapeutic resistance and unfavorable prognosis in HCC. CSCs: Cancer stem cells; HCC: Hepatocellular carcinoma.

The gene set has been shown to predict unfavorable outcomes in patients with these malignancies, which indicates that BMI1-driven pathways are closely associated with an aggressive cancer phenotype.

In a microarray-based gene expression analyses, HCC with similar expression patterns as that of hepatic stem/progenitor cells was associated with poor prognosis[45]. c-MET is a tyrosine kinase receptor for hepatocyte growth factor (HGF). HGF/c-MET signaling plays a crucial role in the development and regeneration of the liver[46]. Kaposi-Novak *et al*[47] conducted global gene expression profiling of wild type and Met-deficient mouse hepatocytes and showed that Met-regulated gene expression signature is associated with aggressive phenotypes in HCC, such as vascular invasion. Similarly, it has been reported that epithelial cell adhesion molecule (EpCAM) served as a surface marker in both hepatic stem cell and CSCs[48]. Furthermore, HCC is subclassified into four groups on the basis of the expression of EpCAM and AFP. These subtypes displayed distinct gene expression patterns with features resembling certain stages of hepatic lineages. $EpCAM^{+}AFP^{+}$ HCCs exhibit hepatocytic progenitor-like expression patterns and have poor prognoses. In contrast, $EpCAM^{-}AFP^{-}$ HCCs exhibit mature hepatocyte-like expression patterns and have favorable prognoses.

Taken together, prognostic stratification based on the expression of surface markers and molecules in hepatic stem/progenitor cells revealed that stemness features are closely associated with unfavorable prognosis in patients with HCC (Figure 2).

BIOLOGICAL MARKERS FOR HCC

Serological markers for HCC

AFP, a plasma protein produced by the yolk sac and fetal liver cells[49], has been the most widely used biomarker for the detection of HCC[50]. However, AFP is not a necessarily specific marker for HCC, considering that its levels of may also be observed in patients with chronic hepatitis and cirrhosis. In contrast, lens culinaris agglutinin-reactive fraction of AFP (AFP-L3) is specific for HCC and has been available in clinical settings[51]. In addition, $AFP\text{-}L3^{+}$ HCC frequently exhibits biologically malignant characteristics, such as portal vein invasion and undifferentiated pathology[52]. Protein induced by vitamin K absence or antagonist-Ⅱ (PIVKA-Ⅱ), also called des-gamma-carboxy prothrombin, is an abnormal prothrombin induced by vitamin K shortage. Hepatocytes with malignant transformation impair the vitamin K-dependent γ-glutamyl carboxylation and produce PIVKA-Ⅱ[53]. The serum levels of PIVKA-Ⅱ as well as AFP in patients with HCC were significantly higher than those in patients with chronic hepatitis and cirrhosis. A marked increase in serum PIVKA-Ⅱ level is also observed in patients receiving anticoagulation therapy with warfarin. Serum PIVKA-Ⅱ level is shown to be closely associated with large tumor diameters and vascular invasion compared with that of AFP and AFP-L3[54]. However, these markers have been shown to be insufficient for the detection of small HCC. Simultaneous measurement of these markers, such as AFP and PIVKA-Ⅱ[55] and AFP-L3 and PIVKA-Ⅱ[56], contributes to the improved diagnostic value for HCC detection.

Recently, dickkopf-1 (DKK1) has been shown to be a promising serum marker for the detection of HCC[57]. DKK1 is a secreted protein with two cysteine-rich regions and functions as a negative modulator of the Wnt/β-catenin pathway by interacting with the co-receptor[58]. It has been shown that serum DKK1 levels were significantly higher in patients with HBV-related HCC than those in the controls[59]. In addition, simultaneous measurement of DKK1 and AFP was shown to improve diagnostic accuracy. Further analyses would be necessary to determine whether DKK1 contributes to the diagnosis of HCV-related HCC.

Molecular markers for HCC

Polycomb group gene products: Polycomb group (PcG) complexes regulate epigenetic cellular memory and establish and maintain cellular identities during embryogenesis, development, and tumorigenesis[60,61]. PcG complexes can be functionally divided into at least two distinct complexes: a maintenance complex, polycomb repressive complex (PRC) 1 and an initiation complex, PRC2. BMI1, one of the components of PRC1,

is essential for maintaining the self-renewal capability of somatic stem cells including hepatic stem cells[62,63]. We have previously shown that BMI1 regulates CSCs in HCC cell lines[64]. These findings suggest that BMI1 regulates self-renewal of both normal stem cells and CSCs by repressing the transcription of negative regulator genes for stem cell maintenance, such as Ink4a and Arf[65]. Furthermore, BMI1 expression levels in HCC tumor tissues are well correlated with the progression and prognosis of the disease[66].

Ezh2, one of the components of PRC2, shows catalytic activity specific for the trimethylation of histone H3 at lysine 27. We have previously reported that Ezh2 tightly regulates the self-renewal and differentiation of murine hepatic stem/progenitor cells[67]. Similar to BMI1, EZH2 is also overexpressed in tumor-initiating HCC cells and HCC tumor tissues[66]. In addition, EZH2-knockdown using short-hairpin RNA and the pharmacological inhibition of EZH2 by an S-adenosylhomocysteine hydrolase inhibitor, 3-deazaneplanocin A (DZNep) markedly impaired the growth and tumorigenic ability of HCC cells[68].

Taken together, PcG proteins such as BMI1 and EZH2 may be encouraging therapeutic targets for HCC. Considering that highly selective PcG protein inhibitors have been developed, clinical trials would be of importance[69,70].

Glypican-3: Glypican-3 (GPC3), a member of the family of glypican heparan sulfate proteoglycans, is an oncofetal protein expressed in fetal liver and HCC[71]. Abnormal expression of GPC3 was observed in approximately 70% of HCC tumor samples and in approximately 50% of serum samples of patients with HCC[72]. Additionally, increased GPC3 expression detected by immunohistochemical analyses correlated with poor prognosis among patients with HC[73]. Considering that a clinical trial using a GPC3 peptide vaccine in patients with advanced HCC has also been carried out[74], this appears to serve not only as a tumor marker but also as a therapeutic target.

Heat shock protein 70: Heat shock proteins (HSPs) are highly conserved protein serve as multifunctional molecular chaperones[75]. Their expression is usually upregulated in response to stressful stimuli such as heat stress. Increased expression of HSP70 has been reported in a wide range of cancers including HCC[76]. Chuma *et al*[77] conducted oligonucleotide array analyses to compare expression profiles among seven pairs of early components and progressed components of nodule-in-nodule type HCCs. They successfully demonstrated that HSP70 expression was upregulated according to the differentiation grade. It is possible that HSP70 could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesions. In addition, combination of markers, such as HSP70, GPC3, and glutamine synthetase[78] and HSP70, GPC3, and EZH2[79] may contribute to the accurate diagnosis of HCC by immunohistochemical analyses.

Sal-like protein 4: Sal-like protein 4 (Sall4), a member of the zinc finger transcription factor family, is highly expressed in murine hepatic stem/progenitor cells and functions as a regulator of cell fate decisions. Overexpression of Sall4 in these cells significantly inhibits hepatocyte-lineage maturation and adversely accelerates cholangiocyte-lineage terminal differentiation[80]. Overexpression of Sall4 in HCC cell lines suppresses hepatocytic differentiation and leads to the acquisition of stem cell-like phenotypes, such as chemoresistance[81]. Together, Sall4 is closely associated with the properties of both normal stem cells and CSCs in liver. Recently, Yong *et al*[82] performed clinicopathological and gene-expression microarray analyses in terms of Sall4 expression and revealed that increased expression of Sall4 was closely associated with aggressive phenotypes of HCC and unfavorable survival. Gene set enrichment analysis showed that Sall4-high HCC were significantly enriched with genes involved in embryonic stem cell signature, metastasis, hepatoblastoma, and progressive HCC compared with Sall4-low HCC. In addition, Sall4 peptide, consisting of 12 amino acids, has been shown to block the oncogenic role of Sall4 in part by modulating PTEN/PI3K/AKT signaling in HCC cells.

Predictive markers for response to sorafenib treatment in HCC

Sorafenib, an oral multi-kinase inhibitor, is available as a new molecular-targeted therapy against HCC. Global guidelines currently recommend sorafenib as first-line therapy for Child-Pugh A patients with advanced HCC[83-86]. Sorafenib demonstrates its anti-HCC effect by suppressing tumor growth factors and angiogenesis through the inhibition of the RAF/MEK/ERK signaling pathway and tyrosine kinase receptors, such as VEGFR[87]. The safety and efficacy of sorafenib on patients with advanced HCC has been demonstrated in phase Ⅲ studies[88,89].

Some investigators have tried to determine the predictive markers for the response to sorafenib, which also serve as predictive indicators of prognosis in patients with HCC. Physical findings, such as hand-foot-skin reaction (HFSR) and hypertension have shown to predict favorable outcomes in patients treated with sorafenib[90,91]. Alternatively, the presence of lung metastasis could predict poor response to sorafenib[92]. It has been also reported that early decrease in AFP level and increase in PIVKA-Ⅱ level determine the therapeutic efficacy of sorafenib[93,94]. Concordant with these findings, our analyses demonstrated that increase in AST or AFP levels, existence of MVI, and lack of HFSR serves as independent predictors of poor prognosis[95]. Recently, it has been reported that genetic amplification of FGF3/4 and VEGF-A was frequently observed in responders to sorafenib in HCC[96,97]. Rudalska *et al*[98] conducted *in vivo* RNAi screening to identify sorafenib-response genes and reported that both shRNA-mediated and pharmacological silencing of MAPK14 (p38α) sensitize

HCC cells to sorafenib therapy. They also highlighted the importance of inhibiting MAPK14-dependent activation of MEK/ERK and ATF2 signaling to overcome sorafenib-resistance.

Taken together, these findings may be useful as prognostic biomarkers and are breakthroughs for understanding of the molecular mechanisms underlying the development and progression of HCC.

CONCLUSION

Progress in molecular biology, such as next-generation sequencing, unveils the biological features of HCC. These analyses were conducted on tissue and blood samples. Recently, the use of "liquid biopsy" to analyze circulating tumor DNA in peripheral blood has been documented[99,100]. This approach is minimally invasive and enables the detection of the sequence and mutations of target genes. Combined use of novel approaches and biomarkers contributes to early diagnosis and the selection of the appropriate treatment for patients with HCC. Further efforts would be needed to improve the prognosis of patients with advanced HCC.

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REVIEW

Optimal combination of antiangiogenic therapy for hepatocellular carcinoma

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Abstract

The success of sorafenib in prolonging survival of patients with hepatocellular carcinoma (HCC) makes therapeutic inhibition of angiogenesis a component of treatment for HCC. To enhance therapeutic efficacy, overcome drug resistance and reduce toxicity, combination of antiangiogenic agents with chemotherapy, radiotherapy or other targeted agents were evaluated. Nevertheless, the use of antiangiogenic therapy remains suboptimal regarding dosage, schedule and duration of therapy. The issue is further complicated by combination antiangiogenesis to other cytotoxic or biologic agents. There is no way to determine which patients are most likely respond to a given form of antiangiogenic therapy. Activation of alternative pathways associated with disease progression in patients undergoing antiangiogenic therapy has also been recognized. There is increasing importance in identifying, validating and standardizing potential response biomarkers for antiangiogenesis therapy for HCC patients. In this review, biomarkers for antiangiogenesis therapy including systemic, circulating, tissue and imaging ones are summarized. The strength and deficit of circulating and imaging biomarkers were further demonstrated by a series of studies in HCC patients receiving radiotherapy with or without thalidomide.

Key words: Antiangiogenesis; Hepatocellular carcinoma; Biomarker; Cytokines; Dynamic contrast enhanced magnetic resonance imaging

Core tip: Antiangiogenic therapy has become an important component of treatment in hepatocellular carcinoma (HCC) patients. However, traditional anatomic imaging of tumor shrinkage is not appropriate to evaluate the efficacy of antiangiogenesis achieved by normalizing tumor vasculature and systemic suppression of angiogenic and inflammatory cytokines. To identify and validate potential response biomarkers, standardized systemic, circulating, tissue and imaging assays should be incorporated in to preclinical and clinical studies regarding the combination of antiangiogenic agents to cytotoxic or biologic agents. The optimal dosage, schedule and duration of antiangiogenic during com-

bination therapy for HCC patients should be titrated according to these response biomarkers.

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INTRODUCTION

Patients with advanced hepatocellular carcinoma (HCC) have a poor prognosis. Systemic therapy with cytotoxic agents provides marginal benefit[1,2]. HCC, a highly vascularized tumor, requires angiogenesis to grow, invade and metastasize[3]. The success of pharmacological inhibition of angiogenesis in HCC provided by sorafenib[4,5], makes it the first and only systemic agent to notably improve survival in HCC patients.

Although the inhibition of angiogenesis in HCC is an established modality of cancer treatment, concerns regarding toxicity and drug resistance still constitute barriers to be overcome. Recent randomized studies comparing multikinase inhibitors - sunitinib[6], brivanib[7], and linifanib[8] - and the combination of sorafenib plus erlotinib[9] with sorafenib alone does not reveal better survival rates or tolerability. In this review, issues brought up by combining antiangiogenic agents with chemotherapy, or other targeted therapy will be summarized. A series of our study, incorporating thalidomide, an antiangiogenic agent, during radiotherapy for HCC patients will be introduced.

STRATEGIES TO IMPROVE ANTIANGIOGENESIS THERAPY IN HCC

To improve the clinical outcomes of antiangiogenic therapy in HCC, the combination of antiangiogenic agents with cytotoxic chemotherapy or with other molecularly targeted therapies may act synergistically to generate additive effects. A better understanding of the mechanisms regarding the action of sorafenib on HCC plus the investigation for predictive biomarkers may allow us to select patients suitable for anti-angiogenesis therapy.

Combinations of anti-Vascular endothelial growth factor (VEGF) agents with chemotherapy in HCC are under evaluation. Treatment with bevacizumab/capecitabine/oxaliplatin or with bevacizumab/gemcitabine/oxaliplatin in HCC patients resulted in median survivals of less than 10 mo[10,11]. Based on the encouraging data from sorafenib plus doxorubicin in HCC[12], a phase Ⅲ randomized study (CALGB80802) comparing sorafenib plus doxorubicin with sorafenib alone is underway in patients with advanced HCC. Studies combining sorafenib with gemcitabine/oxaliplatin, modified FOLFOX, or capecitabine/oxaliplatin are ongoing.

Another approach has been to combine antiangiogenic therapy with inhibitors of other angiogenesis or molecular targets. Tivantinib, a c-MET inhibitor, was compared with placebo in a randomized phase Ⅱ study in advanced HCC. Improved time to progression with tivantinib, especially for patients with tumors of high MET expression was noted[13]. Cabozantinib, a receptor tyrosine kinase inhibitor of c-MET/VEGF receptor 2 (VEGFR2), is undergoing phase Ⅲ evaluation in HCC patients failed or could not tolerate sorafenib[14]. The mTOR inhibitor everolimus was compared with best supportive care alone in a randomized phase Ⅲ trial (EVOLVE-1) in the second-line treatment of HCC patients. No significant survival benefit was noted using everolimus in HCC patients relapsed from sorefinib[15]. Everolimus was also combined with sorafenib in a phase Ⅰ trial of HCC, and 43% of the patients developed grade 3/4 thrombocytopenia[16].

Other inhibitors of genetic or epigenetic targets of HCC including basic fibroblast growth factor (bFGF) inhibitors, heat-shock protein inhibitors, histone deacetylase inhibitors, MEK inhibitors, insulin growth factor (IGF)/IGF receptor inhibitors, Wnt signal inhibitors, immunotherapy with or without the combination of antiangiogenesis are under clinical investigation in advanced HCC[17].

With all the efforts in improving clinical outcomes of antiangiogenesis, the optimal dosing schedule of antiangiogenic agents alone or in combination for HCC patients is largely unclear. Along with the development of new therapies, a parallel effort must be made to identify biomarkers of response, and toxicity in order to provide HCC patients with safe and effective therapies.

RESPONSE BIOMARKER

Anatomic imaging biomarkers that quantify liver tumor response to cytotoxic therapy are based on temporal change in the size of the tumors. Objective response by size-based decrease in tumor, may translate to an early clinical endpoint, in substitution for overall survival[18]. Anti-VEGF therapy has primarily cytostatic effects, may prune and normalize the tumor vasculature, and can have substantial systemic effects such as modulation of circulating proangiogenic and proinflammatory cytokines and cells[19-24]. These effects may not shrink but rather stabilize the tumor size and prolong survival[25,26]. Unlike conventional chemotherapy, an effective dose of an antiangiogenic agent can be less than the maximum tolerated dose, whereas certain toxicities may be dose-related[27]. The development of antiangiogenic therapy or other biologic therapy requires new methods for measuring response to therapy.

Blood pressure as a biomarker

Hypertension has been observed in patients with cancer treated with anti-VEGF antibodies or tyrosine kinase inhibitors (TKIs) and is clinically manageable in most cases with medication. There is evidence that patients

Table 1 Circulating biomarker of hepatocellular carcinoma patients receiving antiangiogenic therapy

Treatment	Patient enrolled	Patient *n*	Predictive value	Prognostic value	Ref.
Sorafenib, bevacizumab or thalidomide/oral 5FU	Elevated AFP	72	AFP responder (AFP decline > 20% in 4 wk) correlate with response	Early AFP responder: associate with PFS, OS	Shao *et al*[103]
Sorafenib	Advanced	30		High baseline IL-8 correlates with PD; high Ang2, G-CSF, HGF, leptin correlate with shorter PFS	Miyahara *et al*[104]
Sorafenib	Advanced	491	High baseline sc-KIT, low HGF correlate with sorefinib response	Baseline Ang2, VEGF, AFP correlate with survival	Llovet *et al*[36]
Sorafenib	Post-operative	29	High phosphor MET correlate with sorafenib resistance		Xiang *et al*[105]
Sunitinib	Advanced	34		High baseline AFP, IL-8, IL-6, SDF1, TNF correlate with PFS and OS; decreased IL-6, sc-KIT at day 14 correlate with improved PFS and OS	Zhu *et al*[24]
Sunitinib	Advanced	37	High baseline VEGFC correlates with response	High base VEGFC correlates with TTP; change in VEGFA, sVEGFR2 correlate with OS	Harmon *et al*[106]
Sunitinib	Advanced	23	Decrease sVEGFR2 or TNF correlate, with decrease in K^{trans} K_{ep}; Decrease K^{trans}, K_{ep} at week 2 correlate with response		Sahani *et al*[107]
Bevacizumab	Advanced	43	Increase CEC on day 15 , low IL-8 correlate with disease control	High baseline IL-8, IL-6 correlate with short PFS, OS	Boige *et al*[108]
Bevacizumab	Advanced	59	High Ang2, EGFR, endothelin 1, no acneiform rash correlate with poor outcome		Kaseb *et al*[109]
Thalidomide	Advanced	47	No predictive value of VEGF, bFGF, PlGF		Hsu *et al*[110]
Thalidomide/tegafur/uracil	Advanced	43		High IL-6, IL-8 correlate with short survival	Shao *et al*[111]
Thalidomide/radiotherapy	Advanced	24	No predictive value of VEGF, bFGF, IL-6, SDF1, TNF	Baseline IL-6, SDF1 at week 2 correlate with PFS. SDF1 at 1 mo post radiotherapy correlates with OS	Ch'ang *et al*[89]
TSU68	Advanced	35	High sVCAM1 correlates with response		Kanai *et al*[112]

AFP: α-fetal protein; PFS: Progression free survival; OS: Overall survival; IL: Interleukin; PD: Progressive disease; Ang2: Angiopoetin 2; G-CSF: Granulocyto colony stimulating factor; HGF: Hepatocyte growth factor; VEGF: Vascular endothelial growth factor; SDF: Stem cell derived factor; TNF: Tumor necrosis factor; TTP: Time to progression; sVEGFR: Soluble VEGF receptor; K^{trans}: Transfer constant; K_{ep}: Redistribution rate constant; CEC: Circulating endothelial cells; EGFR: Epidermal growth factor receptor; bFGF: Basic fibroblast growth factor; PlGF: Placental growth factor; sVCAM1: Soluble vascular cell adhesion molecule 1.

with hypertension have better survival outcomes[28,29]. A significantly improved progression-free survival (PFS) for patients with grade 2/3 hypertension after receiving bevacizumab is noted compared to those who did not develop hypertension on bevacizumab treatment (P = 0.04). These findings suggest the possibility of titrating the dose of anti-VEGF therapy by hypertension for efficacy optimization.

VEGF as a biomarker

The most extensively studied biomarker in antiangiogeni therapy has been VEGF (Table 1). Free VEGFA is rapidly cleared from the circulation, and a wide variation in plasma VEGF concentrations has been reported due to different assay sensitivities[30]. After immune-depletion of VEGF bound to bevacizumab, Loupakis *et al*[31] reported that the concentrations of free and active VEGF decreased significantly from day 0 to day 14 after bevacizumab treatment.

Associations between outcomes of antiangiogenic therapy with VEGF levels in the circulation have been reported in clinical trials of breast cancer and HCC[32,33]. However, in other cancers neither the intra-tumoral nor the circulating VEGF was associated with the outcome of bevacizumab treatment[34,35]. Baseline VEGF and angiopoietin-2 concentrations were found to be independent prognostic markers in the sorafenib HCC assessment randomized protocol trial. However, these biomarkers were not predictive of response to sorafenib[36].

Many studies have shown a lack of correlation between VEGF levels at baseline and the outcome of antiangiogenic therapy[35,37]. Intriguingly, the circulating levels of VEGF seemed to be significantly elevated after most antiangiogenic therapies targeting this pathway[38]. Similar phenomenon was noted after therapy with anti-VEGFR TKIs[39-44]. Preclinical data indicate that this increase in VEGF may be induced by a host-response to hypoxia in tumors.

On the other hand, the VEGF genotype has emerged as a predictive biomarker from the phase Ⅲ study of bevacizumab in metastatic breast cancer (ECOG 2100). VEGF-2578AA genotype was associated with a superior overall survival in the combination arm[45].

Placental growth factor and soluble VEGFRs as biomarkers

Circulating plasma levels of placental growth factor (PlGF) increase consistently in response to anti-VEGF treatment. Thus, plasma PlGF is now being considered as a potential biomarker of anti-VEGF therapy[41,43,46]. Of interest, the increase in PlGF may be due to systemic effects, as tumor-derived PlGF may actually be decreased after bevacizumab treatment[46]. Ziv-aflibercept, a recombinant fusion protein that blocks multiple factors in the angiogenesis network by binding VEGFA, VEGFB and PlGF. Results of the EFC10262-VELOUR study revealed a significant improvement in the primary endpoint of overall survival (OS) with ziv-aflibercept and FOLFIRI compared to FOLFIRI alone[47], despite approximately one-third of the patients having received prior bevacizumab treatment. These findings underscore the potential role of other VEGF family members in tumor angiogenesis.

Circulating levels of soluble VEGFR2 and VEGFR3 proteins are decreased by TKIs that directly target these receptors, but not by bevacizumab. Studies showed that patients with higher plasma levels of sVEGFR1 had a poor outcome after treatment with bevacizumab, sunitinib, vandetanib, and cediranib[24,48-51]. Polymorphisms in the *FLT1* gene that are associated with higher VEGFR1 expression have been associated with poor outcome of bevacizumab containing regimens in phase Ⅲ studies[52]. The mechanisms by which these changes occur, their biological significance, and their utility as predictive biomarkers are not understood.

Other proteins as biomarkers

Collagen Ⅳ is one of the main constituents of tumor vascular basement membranes. Proteolytic degradation of the basement membrane during vascular normalization by antiangiogenic agents can release soluble collagen Ⅳ in blood circulation. In recurrent gliobastoma patients, increase in plasma collagen Ⅳ levels after anti-VEGF therapy was associated with an increase in PFS[53]. In patients with metastatic colorectal cancer, responses to vatalanib plus chemotherapy correlated positively with tissue mRNA levels of VEGFR1, lactate dehydrogenase (LDH) A and glucose transporter (Glut) 1 and inversely with hypoxia inducible factor 1-α[54]. In addition, patients with high baseline serum LDH levels had longer PFS and OS after treatment with vatalanib and chemoradiation[55]. Baseline soluble intracellular adhesion molecule (ICAM)-1 was shown to be an independent prognostic factor of OS in patients treated with bevacizumab and chemotherapy or chemotherapy alone in metastatic non-small cell lung cancer (NSCLC)[37].

Certain inflammatory cytokines might have potent proangiogenic effects. In patients with advanced NSCLC who were treated with vandetanib plus chemotherapy, vandetanib alone or chemotherapy alone, increase plasma VEGF levels for vandetanib monotherapy and increase in plasma interleukin (IL)-8 for combination therapy were associated with increased risk of disease progression[56]. A phase Ⅱ study suggested that IL-8 A-251T polymorphism may be a molecular predictor of response to bevacizumab based chemotherapy in ovarian cancer[57]. In phase Ⅱ studies, the extent of increase in inflammatory cytokines such as IL-10 in the plasma during treatment was associated with an inferior outcome in patients with rectal and ovarian cancer after bevacizumab and chemoradiation treatment, and an inferior outcome in patients with advanced HCC after sunitinib therapy[11,23,46]. Association between increased plasma stem cell derived factor (SDF)1α after treatment and poor outcome in anti-VEGF studies in recurrent glioblastoma, sarcoma and breast cancer were reported[19,58,59]. Increased plasma SDF1α and plasma IL-6 have been associated with poor outcomes in locally advanced rectal cancer and HCC patients treated with bevacizumab, chemoradiation and sunitinib[24,60]. In line with these findings, preclinical studies have shown that sunitinib can induce elevation of circulating inflammatory cytokines in mice, which might result in more aggressive recurrent or metastatic tumors[61,62].

Other circulating factors reported to be associated with clinical outcomes after antiangiogenesis includes plasma angiopoetin-2, bFGF, platelet derived growth factor-BB, soluble Tie2, sICAM-1, and matrix metalloproteinases[24,49,56,63-66] (Table 1).

Tissue biomarkers

Tissue based biomarkers are difficult to establish because of the invasive and costly nature of these procedures and the variations in immunohistochemical procedures and interpretations. Intratumoral levels of VEGF have not been shown to predict survival outcome of anti-VEGF therapy[35,67], although correlations with response rates have been reported[68]. On the other hand, increased SDF1 and CXCR4 were noted in rectal cancer patients after anti-VEGF treatment[58,69]. High carbonic anhydrase IX expression was associated with better tumor shrinkage for metastatic renal cell carcinoma patients treated with sorafenib[70]. Genetic studies of colorectal cancer did not associate *p53*, *KRAS* or *BRAF* mutations with bevacizumab treatment outcome[71]. Single nucleotide polymorphisms (SNPs) in VEGF, VEGFR2 and VEGFR1 were associated with survival after treatment with bevacizumab based regimens[45,52,72]. In line with the important role of inflammatory cytokines in angiogenesis, a consistent finding appeared to be the association between SNPs in *CXCR2* and *IL-8* genes and the outcome after anti-VEGF therapies[57,73,74]. More extensive investigation and validation are warranted to determine a biomarker for antiangiogenesis therapy.

Circulating cells

In response to sunitinib, the number of circulating endothelial cells (CECs) and monocytes can be decreased in patients with HCC and gastrointestinal stromal tumors (GISTs)[22,24]. However, In GISTs

Table 2 Imaging biomarkers of hepatocellular carcinoma patients receiving antiangiogenic therapy

Treatment	Imaging study	Patient *n*	Predictive value	Prognostic value	Ref.
Sorefinib or sunitinib	Perfusion CT, DCEUS	19	⩽ 40% decrease in AUC at 1 mo correlates with PD		Frampas *et al*[113]
Sorafenib	CEUS	21	Reduction in enhancement correlates with response		Moschouris *et al*[114]
Sorafenib	Perfusion CT	10	Increase in MTT correlates with AFP response		Sacco *et al*[115]
Sunitinib	DCEMRI	24	Decreased K^{trans} or K_{ep} correlate PR/SD		Zhu *et al*[24]
Sunitinib	DWI, MRP	23	Decreased K^{trans} or K_{ep} at week 2 correlate with response	High baseline K^{trans} and decrease in EVF correlate with longer PFS	Sahani *et al*[107]
Bevacizumab	Perfusion CT	25	Low baseline MTT correlates with PD; increased MTT correlates with PR/SD		Zhu[2]
Bevacizumab	DCEUS	42	Decrease between day 0-3 of AUC, AUC during wash-in, AUC during wash-out, time to peak intensity correlate with tumor response	Time to peak intensity correlates with PFS; AUC and ACU during wash-out correlate with OS	Lassau *et al*[116]
Bevacizumab/ gemcitabine and oxaliplatin	Perfusion CT, dual-phase contrast enhanced CT	23	High baseline MTT correlates with PR/SD; high baseline K^{trans} correlates with responder	High baseline MTT correlates with better PFS	Jiang *et al*[117]
Bevacizumab	Perfusion CT	22		Reduction in percentage change of FD and low baseline FD correlate with longer OS	Hayano *et al*[118]
Thalidomide	Power Doppler US	47	High baseline vascular index in responder		Hsu *et al*[110]
Thalidomide	Perfusion CT	18	High baseline blood flow and blood volume correlates with progression		Petralia *et al*[119]
Thalidomide/ radiotherapy	DCEMRI	22	High baseline and week 2 Slope in responder	Perfusion parameters over liver parenchyma correlate with PFS and OS	Liang *et al*[87]
Pazopanib	DCEMRI	26	Reductions in IAUGC and K^{trans} not correlate with pharmacokinetic parameters		Yau *et al*[120]

CT: Computed tomography; DCEUS: Dynamic contrast enhanced ulatrasonography; AUC: Area under curve; PD: Progressive disease; CEUS: Contrast enhanced ultrasonography; MTT: Mean transit time; AFP: α-fetal protein; DCEMRI: Dynamic contrast enhanced magnetic resonance imaging; K^{trans}: Transfer constant; K_{ep}: Redistribution rate constant; PR: Partial response; SD: Stable disease; DWI: Diffusion weighted imaging; MRP: Magnetic resonance imaging derived perfusion parameter; EVF: Extracellular volume fraction; PFS: Progression free survival; FD: Fractal dimension; OS: Overall survival; Slope: Initial first-pass enhancement slope; IAUGC: Initial area under the tissue gadolinium concentration-time curve.

patients, clinical benefit was significantly associated with increases in CECs (P = 0.007) as compared with those with progressive disease[22]. TKIs such as cediranib or bevacizumab combined with chemotherapy did not change the amount of circulating progenitor cells. One of the caveats of using CECs as a biomarker is the means of assessment, which needs to be more rigorously established and standardized.

Imaging biomarkers

Noninvasive imaging has been widely applied for monitoring antiangiogenesis therapy in cancer drug discovery (Table 2). The techniques used in molecular imaging include positive emission tomography, single-photon emission computed tomography, molecular magnetic resonance imaging (MRI), optical fluorescence, optical bioluminescence, and targeted contrast -enhanced ultrasound. For example, temporal change in dynamic MRI and computed tomography (CT)-based tissue vascular measures such as blood flow, blood volume, or permeability have been shown to occur after treatment with bevacizumab or anti-VEGFR TKIs in clinical studies[75]. In HCC patients successfully treated with bevacizumab, CT perfusion imaging demonstrated substantial reductions in hepatic tumor blood flow, blood volume and permeability, findings that may predict treatment response[76]. In MRI perfusion studies, HCC nodules treated with sorafenib showed a higher decrease in K^{trans}, which represents the volume transfer constant between blood plasma and the extravascular extracellular space. This finding reflects a decrease in tumor permeability and correlates with longer PFS and OS[77]. The extent of drop in K^{trans} at day 14 after sunitinib in advanced HCC was significantly associated with PFS[24]. The wide spread incorporation of perfusion as a biomarker has been hampered by inconsistencies in quantification results from different software and acquisition methods, as well as the time intensive analysis of data[78,79] (Table 2).

The validation of clinical imaging of angiogenesis will be a slow and costly process. Different types of clinical trials that include histologic analysis will be needed.

CHALLENGES IN IDENTIFYING AND VALIDATING BIOMARKERS

Despite numerous investigations of antiangiogenic biomarkers, no validated biomarkers currently exist for predicting response or identifying appropriate patients for antiangiogenic therapy. Several challenges need to be overcome. Since the mechanisms regarding the actions of the currently approved antiangiogenic agents are not fully understood, there are no adequate criteria of pharmacologic response[80,81]. The development of toxicity or resistance due to the activation of VEGF-independent pathways should also be explored. Besides, the biopsy or blood sample before treatment may not reflect the biology before subsequent treatment. There is also regional heterogeneity with one part of a tumor not necessarily having the same vascularity as another part. A spatially resolved "dynamic biomarkers" are warranted. Furthermore, the measurement of candidate biomarkers should be optimized and standardized before independent validation.

INCORPORATING THALIDOMIDE INTO RADIOTHERAPY FOR HCC: DYNAMIC CONTRAST ENHANCED MRI STUDIES FOR HCC DURING RADIOTHERAPY

With the advancement of modern radiation and respiratory-gating technique, radical radiation to a portion of liver can achieve a high local control rate in patients with advanced HCC[82-84]. However, slow tumor shrinkage and rapid recurrence compromise treatment outcomes. The development of surrogate markers to monitor the response of HCC to radiation is important[85]. The maximal response to radiotherapy is often achieved 6 mo after completion treatment. This slow response makes it difficult to modify an ineffective regimen for HCC in a timely fashion, especially in patients with a low level of serum α-fetal protein. Furthermore, intrahepatic recurrence outside the field of radiation is a common cause of treatment failure[82,83]. Scattered radiation related tissue inflammation and damage may have a deleterious effect on tumor control because of the release of cytokines or angiogenic factors[86].

We evaluated the signal parameters of dynamic contrast enhanced MRI (DCEMRI) over liver parenchuma as well as liver tumor in HCC patients before, during and after radiotherapy. Initial enhancement slope and peak enhancement ratio, representing microcirculation and permeability to contrast material were measured over an operator-defined region of interest. From nineteen patients with advanced HCC, we found that increased signal parameters of the tumor at week 2 during radiation were associated with an improved local response. In the parenchyma, increased signal parameters at week 2 were associated with recurrence or progression[85]. The observation was validated in another forty-three patients. Signal parameters of baseline as well as week 2 during radiotherapy were higher in patients with responsive tumor[87]. Multivariate analysis, however, showed signal parameters over liver parenchyma, but not over tumor, independently predicted PFS and OS[87]. In line with the observation, univariate analysis showed Child-Pugh classification B and poor liver function predicted shorter PFS. These observations emphasized that liver function reserve, but not tumor response, of these heavily pretreated HCC patients impacts the survival after radiotherapy[88].

INCORPORATING THALIDOMIDE INTO RADIOTHERAPY FOR HCC: CYTOKINES AND IMAGE STUDIES

With the a priori DECMRI study in HCC patients receiving radiotherapy, we evaluated the combination effect of thalidomide to radiotherapy within the same population of patients with identical image acquisition and analysis protocols[87,89]. Thalidomide, an angiogenesis inhibitor, was noted to radio-sensitize tumors by reducing interstitial fluid pressure, increase perfusion and tumor reoxygenation[90]. The anti-inflammatory effect of thalidomide could contribute to the radio-sensitization and disease control of HCC[24,91]. Low dose thalidomide resulted in a response rate of less than 10% and a disease stabilizing rate of 50% in HCC patients[92,93]. Twenty-four patients were enrolled and received concomitant thalidomide and radiation. Thalidomide was prescribed at a dose of 100 mg twice daily starting three days before radiotherapy to achieve a steady serum level[94]. The clinical outcomes, cytokine and DCEMRI studies were compared with patients receiving radiotherapy alone. Thalidomide suppressed the serum bFGF significantly and to a lesser extent, the IL-6 and tumor necrosis factor α levels. Multivariate analysis revealed that baseline IL-6 and week 2 SDF1α level independently predicted the PFS. A decreased SDF1α at one month after radiotherapy complete was a significant prognostic factor of longer OS of HCC patients receiving radiotherapy. Patient with responsive or stabilized disease had significant longer OS (288 ± 51 d *vs* 203 ± 52 d, P = 0.02). However, none of the cytokines evaluated correlated significantly with tumor response after radiation. Despite acceptable toxicity and significant suppression of serum bFGF, thalidomide at current dosage and schedule did not correlate with tumor response and survival of HCC patients receiving radiotherapy[89].

On the other hand, DECMRI studies of the 22 HCC patients receiving thalidomide and radiotherapy showed consistently that signal parameters at baseline and at week 2 during radiotherapy correlated with tumor response. However, the addition of thalidomide at current dosage and schedule did not change the signal

parameters significantly compared to the 22 patients receiving radiotherapy only[87]. The inconsistency between serum biomarker and DCEMRI parameter was reported in a study using ribonucleotide reductase M2 inhibitor with radiation in pancreatic cancer patients[95]. In our study, the significant suppression of bFGF by low dose thalidomide may be tumor-independent changes, nonetheless, reflect systemic exposure to thalidomide. They could serve as drug activity markers to determine optimal biological dose ranges, but not as predictive or prognostic biomarkers[20,22]. Our studies indicate that daily dose of 200 mg thalidomide may induce a systemic suppression of angiogenic and inflammatory cytokines. However, the cytokine effect did not translate into vascular change within liver tumor or liver parenchyma. The optimal dosage and schedule of thalidomide during radiotherapy for HCC patients should be further explored.

The superior sensitivity and the lack of radiation put DCEMRI at the forefront of clinical translation as imaging biomarker. However, the analysis of abdominal and thoracic DCEMRI is often impaired by artifacts and misregistration caused by physiologic motion. More recent reports suggested methods available to alleviate post-processing difficulties in DCEMRI for image analysis[96]. DCEMRI parameters seemed to help to predict tumor angiogenesis measured by microvascular density and VEGF expression levels and discriminate malignant from normal tissue[97-99]. A sufficient decrease in tumor vascular parameters was used to assign an appropriate dose for an additional phase Ⅱ trial of an antiangiogenic therapy (AG-013736). The author showed that the day 2 vascular response measured using DECMRI seemed to be a useful indicator of drug pharmacology[100]. However, paradoxical negative correlation between K^{trans} and CD31 expression was reported as well[101,102]. Continuing investigations are needed to accurately depict whether DCEMRI truly has a role in imaging tumor angiogenesis and evaluating response to antiangiogenesis therapy.

CONCLUSION

Recent preclinical and clinical data suggest the advantage of combining antiangiogenic agents with chemotherapy, radiotherapy or other biologic agents in numerous pathologies. However, in order to optimize the effectiveness of the combination, it is essential to study the mechanisms by which antiangiogenesis or strategies over molecular targets are obtained. Standardized systemic, tissue, circulating and imaging biomarkers should be incorporated into well run preclinical and clinical studies, in order to choose the optimal sequence and administration time of these drugs.

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Physical activity as a treatment of non-alcoholic fatty liver disease: A systematic review

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Abstract

AIM: To review the effectiveness of exercise as a therapy for nonalcoholic fatty liver disease (NAFLD) and potential benefits in treating insulin resistance and atherosclerosis.

METHODS: Medline (EBSCOhost) and PubMed were searched for English-language randomized controlled trials and prospective cohort studies in human adults aged $\geqslant$ 18 which investigated the various effects of exercise alone, a combination of exercise and diet, or exercise and diet coupled with behavioral modification on NAFLD from 2010 to Feburary 2015.

RESULTS: Eighteen of 2298 available studies were chosen for critical review, which included 6925 patients. Nine (50%) studies were randomized controlled trials. Five (27.8%) studies utilized biopsy to examine the effects of physical activity on hepatic histology. The most commonly employed imaging modality to determine change in hepatic steatosis was hydrogen-magnetic resonance spectroscopy. Only two studies examined the effects of low impact physical activity for patients with significant mobility limitations and one compared the efficacy of aerobic and resistance exercise. No studies examined the exact duration of exercise required for hepatic and metabolic improvement in NAFLD.

CONCLUSION: While exercise improved hepatic steatosis and underlying metabolic abnormalities in NAFLD, more studies are needed to define the most beneficial form and duration of exercise treatment.

Key words: Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis; Fatty liver; Exercise; Obesity

Core tip: Lifestyle modification through increased physical activity is beneficial in patients with nonalcoholic

fatty liver disease (NAFLD). Although weight loss has been shown to produce improvement in biochemical and histological markers of NAFLD, exercise might improve hepatic steatosis and steatohepatitis even in the absence of major weight loss. Cardiovascular and resistance training both seem to benefit patients with NAFLD; further study is needed to determine if one is more effective than the other. A reduction in sedentary time in the absence of increased intense physical activity might also improve NAFLD, although more research is required.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a burdensome and increasingly prevalent disease throughout the world. It is the most common cause of chronic liver disease among children and adolescents and represents the leading cause of chronic liver disease worldwide[1]. The staggering prevalence of NAFLD, by some estimates affecting more than 30% of the Western population, parallels the increasingly sedentary lifestyle and continued rise of the obesity epidemic[2]. NAFLD is commonly referred to as the liver manifestation of the metabolic syndrome, and risk factors for its development include diabetes, obesity, and hyperlipidemia[3]. The prevalence of NAFLD approaches 90% in patients with hyperlipidemia, 70% in patients with type 2 diabetes, and greater than 91% in patients who undergo bariatric surgery[4-6].

NAFLD encompasses a spectrum of disease ranging from isolated hepatic steatosis to steatosis with inflammation and hepatocyte injury [non-alcoholic steatohepatitis (NASH)], which is an increasingly common cause of cirrhosis and hepatocellular carcinoma and is on trajectory to become the most common indication for liver transplantation in the United States[7]. Patients with concurrent diabetes mellitus are at a higher risk for the development of NASH, particularly as insulin sensitivity worsens. Diabetics with NASH experience higher rates of microvascular complications, such as chronic kidney disease and retinopathy, as well as higher rates of all-cause mortality when compared to non-NASH diabetics[3,8]. In addition, NAFLD is associated with prevalent coronary artery disease and myocardial dysfunction[9]. A diagnosis of NAFLD is an independent risk factor for the development of cardiovascular disease, which represents the leading cause of morbidity and mortality in this patient population[9,10]. Early recognition and treatment of NAFLD is crucial in the prevention of associated cardiometabolic and liver-related mortality.

While numerous pharmacologic agents can be used to treat the metabolic derangements that often coexist in NAFLD, pharmacologic treatments for NAFLD itself are lacking. The first line treatment of NAFLD is lifestyle intervention, including diet and exercise[11,12]. Exercise may aid in the reduction of hepatic steatosis, prevent progression to cirrhosis, and may improve both insulin sensitivity and cardiovascular health, factors which contribute to the leading cause of mortality in this patient population[12]. Despite the well-established benefits of exercise, there is a lack of robust data to support the efficacy of exercise as treatment in NAFLD[13]. Data gleaned from cross sectional studies correlate inactivity and sedentary lifestyles with the development of NAFLD[14,15]. Physical inactivity leads to reduced insulin sensitivity, an increase in visceral and peripheral fat deposition, and an increase in free fatty acid uptake by the liver[16]. Exercise as a treatment for NAFLD targets many aspects of the disease: the metabolic syndrome, insulin resistance, hepatic steatosis, and cardiovascular disease. Exercise also thwarts the proposed two step development of steatohepatitis, which occurs as a result of deranged fatty acid and lipid metabolism, leading to increased deposition and impaired export of hepatic lipids along with *de novo* lipogenesis, followed by an increase in inflammatory cytokines and infiltrate within the liver[17,18].

Current guidelines do not address specific recommendations for exercise therapy among persons with NAFLD, such as which form of exercise, level of intensity, or duration of treatment provides the most benefit for NAFLD reduction. Well-designed studies in diabetic populations, a population that shares a similar physiology with NAFLD, suggest that combination exercise with both aerobic and resistance exercise achieves the greatest improvement in metabolic parameters including glucose control and abdominal adiposity, and it reduces the risk of developing cardiovascular disease and the microvascular complications of diabetes[19,20]. The aim of the current study is to conduct a systematic review of the available published literature to assess the efficacy of exercise as a treatment for NAFLD and its effect on the cardiometabolic comorbidities of NAFLD, including insulin resistance, dyslipidemia and amount of visceral adiposity. Various forms of physical activity treatment will be reviewed, including exercise programs with and without controlled diets and exercise of varying intensity, duration, and form.

MATERIALS AND METHODS

This systematic review adheres to the relevant criteria from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement. The methods used, including identification, screening, eligibility, and inclusion, were agreed by the authors (Whitsett M and VanWagner LB) in advance. An electronic search of the English language medical literature was conducted using Medline (EBSCOhost) and PubMed to identify

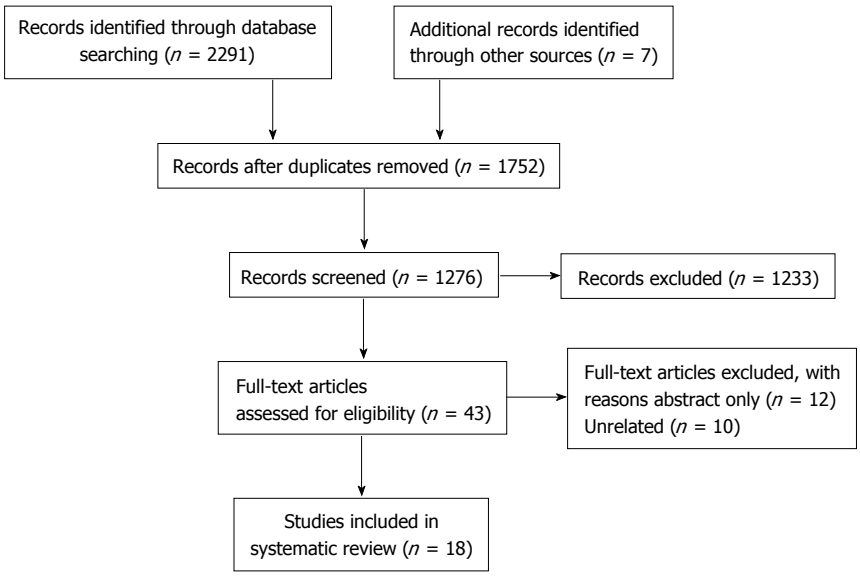

Figure 1 Preferred Reporting Items for Systematic Reviews diagram.

published articles on the role of physical activity as a treatment for NAFLD in adults aged ⩾ 18 years of age. This search strategy used a combination of the following prespecified MeSH headings and keywords alone or in combination: "NAFLD", "nonalcoholic fatty liver disease", "fatty liver", "hepatic steatosis", "NASH", "nonalcoholic steatohepatitis", "non-alcoholic steatosis", "exercise", "resistance training", "aerobic training", "aerobic exercise", "circuit training", "walk test", "endurance training", "strength training", "weight training". Boolean operators ("and", "or") were also used in succession to narrow or widen the search. The search was restricted to English language and human studies.

Inclusion criteria

Studies examining the association between physical activity in adult patients with NAFLD were included. Randomized controlled trials, prospective cohort trials, and well-constructed retrospective studies were included.

Exclusion criteria

Non-English language studies and animal studies were excluded. Studies which examined adolescents or children (age < 18) were excluded.

RESULTS

A total of 2298 studies were initially identified through a comprehensive database search. An additional 7 studies were identified from a hand review of references. After duplicate removal and screening for studies published from 2010 until February 2015, 1276 studies were identified. Fifty-three relevant studies were screened through review of article title and abstract, and eighteen studies were included in this review (Figure 1). Nine (50%) of these studies were randomized controlled trials. A total of 6925 patients were included in these studies. Five (27.8%) studies utilized biopsy to examine the effects on hepatic histology, and the most commonly employed imaging modality to determine change in hepatic steatosis was hydrogen-magnetic resonance spectroscopy (H-MRS). Two studies examined the effects of low impact physical activity for patients with significant mobility limitations. One study compared the efficacy of aerobic and resistance exercise in NAFLD patients. Study characteristics are summarized in Table 1. Main study findings will be discussed below and are summarized in Table 2.

Diet and exercise

Eckard *et al*[21] examined how variations in diet with moderate intensity exercise regimens impact the NAFLD activity score (NAS), which evaluates the degree of hepatocellular ballooning, steatosis, and inflammatory infiltrate on liver biopsy[22]. He enrolled 56 participants in four distinct groups: (1) standard control (n = 14); (2) low fat diet with moderate exercise (n = 14); (3) moderate fat/low processed carbohydrate diet with moderate exercise (n = 13); and (4) moderate exercise only (n = 15). For six months, participants in the exercise arms engaged in an 18-step exercise program that combined aerobic and resistance exercises for 20-60 min, 4-7 d per week. Participants assigned to the low fat and moderate fat groups received nutritional instruction, attended special nutrition classes, and received personalized diet plans, designed with a goal of achieving one pound of weight loss per week based on caloric intake and energy expenditure. All participants received instructions on the completion of a 3-d food log. Participants in the standard care group also attended one specialized nutrition class. Participants in the low fat and moderate fat groups received nutritional follow-up counseling and specific dietary education. Biopsies were performed before and after the intervention

Table 1 Characteristics of published studies which assess the relationship between physical activity and nonalcoholic fatty liver disease (2010-2015)

Ref.	*n*	NAFLD assessment	Design	Parameter studied	Type of activity intervention	Nutrition intervention	Exercise			
							Frequency	Intensity	Duration	Intervention duration
Hallsworth *et al*[26]	37	H-MRS	P	Sedentary and physical activity time	Activity monitor	No	NA	NA	NA	7 d
Gerber *et al*[14]	1263	Fatty liver index > 60	C	Level of physical activity	Activity monitor	No	NA	NA	NA	7 d
Oh *et al*[33]	18	US, H-MRS	P	Hepatic steatosis	Acceleration	No	2 × wk	NA	20 min	12 wk
Kawaguchi *et al*[32]	35	US	P	Hepatic steatosis	Hybrid	Yes	2 × wk	NA	20 min	12 wk
Kistler *et al*[34]	813	Liver biopsy	R	Physical activity, NAS	None (self-report)	No	NA	Inactive, moderate, vigorous	NA	NA
Haus *et al*[36]	17	H-MRS	P	IR, intrahepatic TG content	Aerobic	No	Consecutive days	80%-85% max heart rate	50-60 min	7 d
Promrat *et al*[38]	31	Liver biopsy	RCT	NAS	Aerobic	Yes	Weekly	Moderate	200 min	48 wk
Pugh *et al*[39]	13	US, liver enzymes	RCT	Cutaneous microvascular function	Aerobic	No	3 × wk	Moderate	30-45 min	16 wk
Pugh *et al*[40]	34	US, liver enzymes	RCT	Microvascular function	Aerobic	No	3 × wk	Moderate	30-45 min	16 wk
Sullivan *et al*[27]	18	H-MRS	RCT	Intrahepatic TG content, lipid kinetics	Aerobic	No	5 × wk	Moderate	30-60 min	16 wk
Jin *et al*[23]	120	Liver biopsy	R	Steatosis by histology	Aerobic	Yes	3 × wk	NA	20 min	No set length
Oh *et al*[28]	52	US	P	Hepatic steatosis	Aerobic	Yes	3 × wk	Max HR > 40%	90 min	3 mo
Sun *et al*[24]	1087	US, liver enzymes	RCT	Metabolic parameters	Aerobic	Yes	NA	NA	NA	12 mo
Zelber-Sagi *et al*[30]	82	US	RCT	Hepatic steatosis	Resistance	No	3 × wk	NA	40 min	3 mo
Hallsworth *et al*[29]	21	H-MRS	RCT	Intrahepatic lipid content	Resistance	No	3 × wk	NA	45-50 min	8 wk
Bacchi *et al*[43]	31	MRI	RCT	Hepatic steatosis	Resistance and aerobic	No	3 × wk	Moderate	60 min	4 mo
Eckard *et al*[21]	41	Liver biopsy	P	Histology	Resistance and aerobic	Yes	4-7 × wk	Moderate	20-60 min	6 mo
Oh *et al*[35]	169	US	RCT	Hepatic steatosis	Aerobic	Yes	Weekly	Vigorous	150->250 min	12 wk

NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; DM: Diabetes mellitus; US: Ultrasound; P: Prospective; R: Retrospective; C: Cross-sectional; RCT: Randomized controlled trial; NAS: NAFLD activity score; IR: Insulin resistance; TG: Triglycerides; NA: Not assessed; HR: Heart rate; H-MRS: Hydrogen-magnetic resonance spectroscopy; MRI: Magnetic resonance imaging.

to determine the change in NAS. All groups experienced a significant decrease in NAS from baseline, yet there was no statistically significant difference between groups. Additionally, there was a significant decrease in aminotransferase levels. Not one group achieved a significant degree of weight loss. Of note, there was a high attrition rate in this particular study, with a loss of fifteen participants. Additionally, 50% of participants in both the low fat and moderate fat groups were non-adherent to their prescribed diet and caloric expenditure. Because of the complexity of the diet intervention as well as the poor adherence to the prescribed medium and low fat diets, it is difficult to ascertain what effect, if any, dietary changes had on NAFLD. However, this study suggests that exercise without weight loss can still achieve an improvement in the health of NAFLD patients.

Jin *et al*[23] performed a retrospective study to determine how aerobic exercise and lifestyle changes improved steatosis in 120 non-obese potential living liver donors found to have NAFLD on routine biopsy. Because graft survival after liver transplantation is heavily impacted by the degree of hepatic steatosis of the donor, it is imperative that donors maintain healthy lifestyles and engage in physical activity prior to donation. Patients originally biopsied and found to have moderate to severe steatosis (greater than 30%-60% hepatocytes on biopsy with fat granules) or mild steatosis (5%-30%) with graft-to-recipient weight ratio < 0.8 were encouraged to reduce total body weight by 5% through diet and aerobic exercise. No patients had fibrosis or NASH within the trial. The regimen consisted of aerobic exercises, thrice weekly for at least 20 min per session, and the diet recommended was 25 cal/kg × ideal body weight (kg) consisting of 50% carbohydrates, 20% protein, and 30% fat. Patients were re-biopsied according

Table 2 Published study findings for the relationship between physical activity and nonalcoholic fatty liver disease (2010-2015)

Ref.	Population	Type of activity intervention	Weight loss (% Δ)	Insulin resistance and lipids	Inflammation and oxidative stress	Liver enzymes	Liver fat by imaging	Liver histology	Conclusions
Hallsworth *et al*[26]	NAFLD	Activity monitor	NA	NA	NA	NA	NA	NA	NAFLD = more sedentary time, less energy expenditure, and greater prevalence of DM than healthy controls
Gerber *et al*[14]	NAFLD, NAFLD + DM	Activity monitor	NA	NA	NA	NA	NA	NA	NAFLD = less PA time than non-NAFLD NAFLD + DM = lowest quartile of average PA as well as moderate-vigorous PA
Oh *et al*[33]	NAFLD	Acceleration	-1.9%	NA	↓ TNF-α, IL-6, leptin, IMCL ↑ adiponectin	Improved	Improved (US)	NA	Acceleration training results in significant improvement in IR, inflammation, liver enzymes, steatosis and quality of life
Kawaguchi *et al*[32]	NAFLD	Hybrid	-0.9%	↓ Insulin HOMA-IR	↓ IL-6	Improved	Improved (US)	NA	Hybrid training results in significant improvement in IR, inflammation, liver enzymes and steatosis
Kistler *et al*[34]	NASH, NAFLD	None (self-report)	NA	↓ Insulin and glucose (vigorous PA *vs* inactive)	No effect	No effect	NA	Vigorous PA = ↓ odds of NASH and fibrosis	Vigorous but not moderate or total exercise is associated with the severity of NAFLD
Haus *et al*[36]	NAFLD	Aerobic	0	↓ Glucose HOMA-IR	↑ Lipid PUI, adiponectin	NA	NA	Improved steatosis	Short-term aerobic exercise favorably alters hepatic lipid composition by increasing polyunsaturated lipids
Promrat *et al*[38]	NASH	Aerobic	-9.3%	↓ Glucose, insulin, HbA1C HOMA-IR (NS)	NA	Improved	NA	Improved NAS. No change in fibrosis	> 7% weight loss resulted in improvement in overall NAS, ballooning, steatosis, inflammation
Pugh *et al*[39]	NAFLD	Aerobic	0	No effect	No effect	Improved	No effect	NA	Aerobic exercise improves NO-mediated vasodilation in NAFLD
Pugh *et al*[40]	NAFLD	Aerobic	0	↓ Glucose	No effect	No effect	No effect	NA	Aerobic exercise improves flow mediated dilation in NAFLD
Sullivan *et al*[27]	NAFLD	Aerobic	0	No effect	No effect	Improved	Improved (H-MRS)	NA	Aerobic exercise without weight loss results in significant reduction in intrahepatic triglyceride content
Jin *et al*[23]	NAFLD	Aerobic	-3.9%	↓ Total cholesterol	NA	Improved	NA	Improved steatosis	Aerobic exercise results in decreased steatosis among living donors even in the absence of significant weight loss
Oh *et al*[28]	NAFLD	Aerobic	-13.3%	↓ HbA1C HOMA-IR, LDL, TG ↑ insulin, HDL	↓ TNF-α, IL-6, leptin, hsCRP, ferritin, TBARS ↑ adiponectin	Improved	Improved (US and Fibroscan)	NA	Diet with exercise exceeds diet alone in reducing steatosis, inflammation, insulin resistance
Sun *et al*[24]	NAFLD	Aerobic	-11.6%	↓ HOMA-IR, total cholesterol	No effect	Improved	NA	NA	Aerobic exercise results in decrease in ALT, insulin resistance, and the metabolic syndrome
Zelber-Sagi *et al*[30]	NAFLD	Resistance	-0.75%	↓ Total cholesterol	↓ Ferritin	No effect	Improved (US)	NA	Resistance exercise results in reduction in steatosis, abdominal adiposity, inflammation, cholesterol
Hallsworth *et al*[29]	NAFLD	Resistance	0	↓ HOMA-IR (NS)	↑ Fat oxidation	No effect	Improved (H-MRS)	NA	Resistance exercise results in a 13% relative reduction in intrahepatic lipids
Bacchi *et al*[43]	NAFLD + DM	Resistance *vs* aerobic	No	↓ HDL, TG, HbA1c ↑ clamp-measured insulin sensitivity	No effect	No effect	Improved (MRI)	NA	Both resistance and aerobic exercise result in improved steatosis, abdominal, and visceral fat content

Eckard *et al*[21]	NAFLD, NASH (88%)	Resistance and aerobic	-1.3%[1]	No effect	No effect	Improved	NA	Improved NAS in all groups	Lifestyle modification, even without weight loss, improves NAS
Oh *et al*[35]	NAFLD	Aerobic	10.4%[2]	↓ HOMA-IR, LDL, TG ↑ HDL	↓ TNF-α, IL-6, leptin, hsCRP, ferritin, TBARS ↑ adiponectin	Improved	Improved (US, fibroscan)	NA	Moderate to vigorous PA (> 250 min weekly) significantly reduces markers of IR, oxidative stress and fatty acid metabolism independent of weight reduction

[1]Weight lost in the diet plus exercise arms, mild weight gain was seen in the moderate exercise alone arm; [2]Average weight lost across all groups (range 6.4%-12.4%). ALT: Alanine aminotransferase; DM: Diabetes mellitus; H-MRS: Hydrogen-magnetic resonance spectroscopy; HbA1C: Glycosylated hemoglobin; HDL: High-density lipoprotein cholesterol; HOMA-IR: Insulin resistance by homeostasis model assessment; hsCRP: High sensitivity c-reactive protein; IL-6: Interleukin-6; IMCL: Intramyocellular lipids; LDL: Low-density lipoprotein cholesterol; NA: Not assessed; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NO: Nitric oxide; NS: Not statistically significant; PA: Physical activity; PUI: Lipid polyunsaturated index; TBARS: Thiobarbituric acid reactive substances; TG: Triglycerides; TNF-α: Tumor necrosis factor alpha; US: Ultrasound.

to the health status of the recipient or once they had achieved a loss of 5% or more of their total body weight. Eighty-five percent of donors (103 of 120 patients), including some who maintained the same weight or lost none, experienced an improvement in steatosis. In total, the group experienced a 21.3% reduction in total steatosis. Thus, not only can steatosis improve significantly when diet and exercise lead to weight loss, but patients who partake in aerobic exercise and diet without experiencing weight loss can still achieve improvement in steatosis.

Sun *et al*[24] launched an impressively large study in China by studying the effect of lifestyle intervention on various metabolic parameters in 1087 NAFLD patients over the course of one year. Patients were randomized to control or lifestyle; the lifestyle intervention group was given a special diet: 30% fat, 15% protein, and 35% carbohydrates. They were encouraged to walk, jog, stair climb, and do other exercises with a goal of 23 metabolic equivalent tasks (METs)•h/week (physical activity) + 4 METs•h/week (exercise). Those in the control group were encouraged to maintain their everyday habits and were provided education on the value of healthy eating and exercise in NAFLD. Patients in the lifestyle arm experienced a significant reduction in alanine aminotransferase (ALT), insulin resistance, and prevalence of the metabolic syndrome when compared to controls. While the study showed that the group undergoing lifestyle intervention experienced a significant improvement in their health, it is difficult to ascertain the true benefit of exercise with the lack of controlling for diet.

Exercise interventions

Activity levels in patients with NAFLD: Living a sedentary lifestyle can lead to the development of obesity and insulin resistance, factors which then can predispose to the metabolic syndrome. Sedentary time is defined as periods of both lengthy periods of inactivity and lack of movement, such as in television watching, sleeping, or sitting[25]. Previous studies gauging sedentary behavior in NAFLD patients relied on subjective data, surveys mostly, to reach their conclusions. Hallsworth *et al*[26] objectively measured the extent of physical inactivity and sedentary time in NAFLD patients. He provided wearable devices to all participants, which measured activity level and energy expenditure over the course of seven days. As expected, NAFLD patients reported having longer periods of sedentary time, less energy expenditure, and less walking time and active time than healthy controls. While the NAFLD patients had on average a higher magnetic resonance imaging (BMI) and weight, no comparison could be made regarding the degree of hepatic steatosis or insulin resistance between case and control due to the fact that only NAFLD patients received laboratory evaluation. Gerber *et al*[14] gleaned data from the National Health and Nutrition Examination Survey from 2003-2004 and 2005-2006 to examine 3 groups: NAFLD alone, NAFLD plus diabetes, and healthy controls. He assessed the difference in physical activity between the groups by the use of specially designed activity monitors. NAFLD patients were found to be less physically active, have increased sedentary time, and have a modest prevalence of diabetes. Neither study offered any exercise intervention *per se*, but it is important to note that in addition to encouraging formal exercise, minimizing sedentary time may be another approach to minimize the deleterious effects of NAFLD.

Aerobic activity and NAFLD: Sullivan e*t al*[27] sought to examine the relationship between aerobic exercise and hepatic lipid levels, hypothesizing that moderate intensity aerobic exercise would not only reduce triglyceride content within the liver but that it would decrease the rate of secretion of very low density lipoprotein triglycerides (VLDL-TG) by the liver. Eighteen patients were divided into the exercise arm, which required exercising at 45%-55% peak oxygen consumption (VO_2) rate

for 16 wk with 30-60 min sessions held 5 times weekly. There was not a dietary component to either arm of the study, and control patients were told to continue with their daily activities. Patients underwent H-MRS pre and post intervention to evaluate the triglyceride content of their livers, and underwent a study with isotope tracer to determine if the VLDL secretion rates had improved. After the intervention, the concentration of intrahepatic triglyceride content in the exercise arm decreased significantly (> 10%), without a significant change in body composition or weight. The reduction of hepatic triglycerides also correlated with a reduction in serum ALT. Exercise had no significant effect on VLDL-TG or VLDL-apoB100 secretion rates or lipid plasma concentration. While the study evaluated a small number of patients, the results bolster the body of evidence that suggests that exercise with a moderate level of intensity has a modest impact on the degree of hepatic steatosis in NAFLD patients.

Oh *et al*[28] studied how a regimented diet in conjunction with aerobic exercise can improve hepatic steatosis and various anthropometric parameters, such as visceral and subcutaneous adiposity, when compared to diet alone. Fifty-two obese men with NAFLD either engaged in diet alone or diet plus aerobic exercise which consisted of either walking or jogging for 90 min thrice weekly at a maximum heart rate > 40%. The diet was 1680 kcal daily, and patients kept food journals, met with dietitians, and attended group education sessions. The diet and exercise group experienced a greater improvement in inflammatory serum markers as well as a greater reduction in hepatic steatosis, visceral and subcutaneous adiposity, and insulin resistance compared to patients who only dieted. Cardiorespiratory fitness, as measured by VO_{2max}, improved in both arms but to a greater degree in the combined diet and exercise cohort. Also, there was a significant correlation between the volume of exercise (measured by the change in number of steps) and the degree of reduction of steatosis, suggesting that greater duration of exercise produces an appreciable difference in markers of hepatic function. Thus, diet coupled with exercise has an overall greater benefit than diet alone in improving body habitus and markers of inflammation and oxidative stress in NAFLD patients.

Resistance training and NAFLD: Resistance exercise may be more feasible for certain subgroups of NAFLD patients, particularly for those with poor cardiorespiratory fitness or those who are overweight and cannot, due to body habitus, tolerate or participate in aerobic fitness. Hallsworth *et al*[29] examined the effect of resistance training without weight loss on NAFLD patients with sedentary lifestyles, defined as less than 60 min of vigorous activity daily. The study did not include dietary intervention, and the diets of the participants were unknown. After eight weeks of a structured exercise program targeting various muscle groups and with progressive increase in the amount of resistance, researchers found that even in those patients who did not lose weight, the resistance exercise group had a significant reduction in hepatic steatosis as well as an improvement in glycemic control and lipid oxidation. In both the control and resistance groups, BMI remained relatively stable, and there was no significant change in ALT or lipids. Thus, in NAFLD, it is possible to achieve improvement in hepatic steatosis, insulin resistance, and lipid oxidation without losing weight. Therefore, patients with functional limitations or poor cardiorespiratory fitness who may struggle with the demands of aerobic exercise can still benefit from resistance training.

Zelber-Sagi *et al*[30] sought to investigate the effect of resistance training on NAFLD in reducing hepatic steatosis, as measured by ultrasound and the hepatorenal index, a ratio which compares the echogenicity of the liver and kidney to quantify the degree of hepatic steatosis. This form of exercise was compared to home stretching exercises[31]. Patients in the treatment arm completed a three-month program comprised of resistance exercises with the goal of progressively increasing the intensity of resistance. The workouts were self- monitored and specifically avoided aerobic exercise. The stretching arm was provided stretching exercises targeted to eight different muscle groups; stretching was performed three days per week. There were no dietary restrictions, and at the start and completion of the intervention patients provided information regarding their nutritional intake.

Zelber-Sagi *et al*[30] reported that there was a significant reduction for the resistance arm in the hepatorenal index (11% *vs* 3.5%), a significantly greater reduction in hepatic and abdominal adiposity as well as a decrease in serum ferritin and cholesterol compared to the stretching group. Thus, not only is resistance exercise an important adjunct to aerobic exercise in treating steatosis, but it seems to help mitigate aspects of the inflammatory environment, which are thought to contribute to the pathogenesis of cardiovascular disease and progressive steatohepatitis among NAFLD patients.

Exercise training in NAFLD patients with physical limitations: Hybrid and acceleration training

There is a subset of NAFLD patients in whom moderate aerobic or resistance exercise is exceptionally difficult (the morbidly obese, incapacitated or bedridden, elderly, or those with other mobility-limiting comorbidities). More rigorous forms of exercise may also be unsafe for patients to complete. Hybrid training consists of simultaneous voluntary muscular contraction and electrical stimulation of the opposing muscle group. Kawaguchi *et al*[32] examined the efficacy of hybrid training to improve the metabolic consequences of NAFLD. In this study, patients performed both knee flexion and extension exercises. Hybrid training does not require a patient to be standing, thus these exercises can be performed while in bed and could potentially be of benefit to patients with low mobility. All 35 patients enrolled received 12 wk of nutritional counseling. Patients in the control group were

advised to consume less than 25% of total calories from fat and consume a diet consisting of 25-30 kcal/kg per ideal body weight. Those in the hybrid group had 24 total exercise sessions, twice weekly for 12 wk. There was a significant decrease in body weight, body fat, serum ALT, hepatic steatosis, and insulin resistance. There was no effect on serum lipids or basal metabolic rate. Low intensity exercise, such as hybrid training, offers promise to NAFLD patients who are debilitated by their illness or other comorbidities.

Another type of exercise that can be used in relatively immobile patients is acceleration training. Acceleration training is a new training method that provides a physical stimulation effect on skeletal muscles by increasing gravitational acceleration with vibration. Participants either hold particular poses or engage in dynamic movements to activate muscle fibers and increase muscular endurance and strength. Oh *et al*[33] studied the effect of acceleration training on obese patients with NAFLD who previously struggled with weight loss in another study. Eighteen obese NAFLD patients who had completed a 12-wk counseling program for lifestyle changes without experiencing an improvement in hepatic enzymes or steatosis were chosen for this study. The exercise program consisted of acceleration training and utilized whole body three dimensional vibration on a special platform. There was no specific diet for this study; however, patients did receive dietary education as well as keep a food log for three days. At the end of the 12 wk of training, there was a significant improvement in anthropometry and intramyocellular lipid content. Intrahepatic steatosis decreased by 8.7%. Also notable were the reported improvements in quality of life and mental health of the patients after the intervention, which factors positively into motivation and willingness to make lifestyle changes. While this is a relatively new form of exercise explored in NAFLD, it may be a promising alternative to traditional exercise in certain subpopulations of NAFLD.

Optimal frequency, intensity, and duration of exercise

For those patients who can exercise without limitations, questions remain over what frequency, intensity, and duration is sufficient to improve features of NAFLD. Kistler *et al*[34] performed a retrospective analysis of self-reported physical activity levels and sought to explore the association between the histopathology of NAFLD and the volume and intensity of the reported exercise regimen. The authors posited that individuals who met moderate to vigorous exercise recommendations would have less fibrosis on pathology and have a lower frequency of NASH. Researchers examined survey results and correlated these with liver biopsy pathology of 813 adults with NAFLD enrolled in two trials from the NASH Clinical Research Network. Patients reported the volume, type, and intensity level of exercise as measured by metabolic equivalent values. No dietary intervention occurred in either of the trials. A large proportion of these patients did not achieve adequate volume or intensity of exercise, with 54% of those polled reporting inactive lifestyles. Those who reported vigorous activity (26% of patients) had lower serum insulin, gamma-glutamyl transferase, and glucose levels. Additionally, patients fulfilling the minimum requirements for vigorous activity experienced a significant reduction in their adjusted odds of having NASH (OR = 0.65, 95%CI: 0.43-0.98, P = 0.04). Those exceeding minimum requirements had a significantly lower odds of having advanced fibrosis (OR = 0.56, 95%CI: 0.34-0.90, P = 0.02). Thus, the intensity of exercise had a greater role in improving NAFLD than the volume or duration of intervention.

Oh *et al*[35] examined the benefits of varying degrees of intensity in exercise programs and how the intensity level impacted the degree of hepatic steatosis. Patients were divided into three groups: exercise < 150 min/wk, 150-250 min/wk, > 250 min/wk. The patients exercised for 12 wk total and were instructed to remain on a strict diet of 1680 kcal daily. A uniaxial accelerometer was used on patients to measure energy expenditure. At the study's completion, all groups experienced a significant reduction in weight and BMI. Patients exercising > 150 min weekly achieved a reduction in weight of 12.4% as well as an improvement in hepatic steatosis. Those who exercised for 250 min or longer per week experienced an improvement in hepatic steatosis, ferritin, and other inflammatory markers. Thus, moderate to vigorous exercise of 250 min weekly provided anti-oxidative and anti-inflammatory benefits to NAFLD patients.

To explore the optimal duration of exercise, Haus *et al*[36] investigated the role of short duration (< 7 d) exercise in affecting hepatic steatosis. Researchers hypothesized that even short periods of aerobic exercise could be beneficial in NAFLD by leading to a change in lipid composition of the liver, reducing pro-inflammatory substances, and improving insulin sensitivity[37]. Seventeen obese, non-diabetic NAFLD patients completed a 7-d course of aerobic exercise comprised of walking or jogging for 50-60 min daily at 80%-85% maximum heart rate. H-MRS scans were performed before and after the intervention. Researchers demonstrated that there was an increase in polyunsaturated lipid content in the liver post-exercise. Also, they observed an increase in serum adiponectin, an anti-inflammatory molecule that regulates lipid oxidation. The findings of this study are consistent with known benefits of exercise: improvement in insulin sensitivity and a reduction in the formation of damaging reactive oxygen species.

Combination of diet, exercise, and behavioral modification

Most of the studies reviewed in this paper contain a wide range of patients within the spectrum of fatty liver disease, but the most at-risk group for poor outcomes are those with NASH. Promrat *et al*[38] recruited patients with biopsy-proven NASH to determine if achieving a loss of 7%-10% of body weight through behavioral modification, exercise, and diet would achieve histologic

improvement of NASH[39]. Researchers randomized NASH patients to either exercise or a control group for 48 wk. The control group received education on lifestyle modification, diet, and exercise. The lifestyle intervention arm combined behavioral strategies, individualized diets, and moderate intensity exercise. The caloric allotment in the diets was based upon a patient's starting weight, and the goal was to achieve a 0.5-1 pound weight loss weekly. Patients chose their own exercise and were encouraged to walk 10000 steps daily and achieve 200 min weekly of exercise. On biopsy, the lifestyle intervention group had a significantly larger improvement in NAS. Both groups experienced improvement in ballooning score, and neither group experienced a change in fibrosis score. Those who lost greater than or equal to 7% total body weight had a significant improvement in NAS, hepatic steatosis, ballooning, and inflammation. The lifestyle intervention group experienced a significantly larger mean weight change (-8.7 kg *vs* -0.5 kg) and percentage weight reduction than controls at 24 and 48 wk. The fact that a number of patients (67% *vs* 20%, $P = 0.02$), enrolled in the intervention arm experienced a complete resolution of NASH on biopsy is important, as these patients are at high risk of adverse clinical outcomes if not treated promptly. However, exercise was again combined withdiet and behavioral modification so the incremental effect of moderate intensity exercise cannot be elucidated from this trial.

Effect of physical activity on non-liver outcomes: Insulin resistance and cardiometabolic disease markers among NAFLD patients

Because the leading cause of morbidity and mortality among patients with NAFLD is due to cardiovascular disease, researchers are interested in understanding how exercise mitigates the factors present in NAFLD that predispose to atherosclerosis. An impairment in the body's ability to properly regulate microvascular circulation occurs early in the atherogenic process. Pugh *et al*[40] examined how exercise impacts the microvascular function of cutaneous vessels through measuring the release of nitric oxide (NO) in NAFLD patients. NO is an important mediator in vasodilation during exercise, and it is known that a deficiency of NO contributes to inflammation and lipid deposition within vessel walls. To assess patients' cutaneous blood flow, researchers placed microdialysis probes into cutaneous vessels. Doppler probe signals generated then allowed the researchers to calculate cutaneous vascular conductance. Fourteen patients with NAFLD were assigned to either fully-supervised exercise training or conventional group which received information on lifestyle modification and encouragement to exercise. The exercise arm participated in moderate intensity exercise, three times weekly at 30% of heart rate reserve. The patients eventually escalated to five sessions per week of 45 min duration. Researchers found that as the patients' skin heats from exercise, they experienced increasing amounts of NO release and vasodilation. This improved with time as patients progressed in their exercise routines. These findings suggest that exercise of any sort may help to reduce the risk of cardiovascular disease by improving microvascular function throughout the body.

Pugh *et al*[40] then sought to establish a relationship between NAFLD and endothelial function[41]. Additionally, researchers also wanted to determine if the amount of visceral and hepatic fat would correlate with the degree of endothelial impairment. Endothelial dysfunction is a prerequisite for the development of atherosclerosis and subsequent cardiovascular disease[42]. Endothelial function was measured by flowmediated dilation (FMD) of the brachial artery in 21 patients with NAFLD. Thirteen patients participated in moderate intensity exercise, consisting of supervised and individualized programs. At initiation, patients exercised for 30 min weekly three times per week at 30% of heart rate reserve and then progressed to 45 min sessions five times weekly. There was no specific diet that patients followed. The control group received teaching sessions on exercise and healthy eating, and these patients were permitted to exercise if they wanted. There was a significant improvement in FMD among the exercise group (3.6%, 95%CI: 1.6-5.7, $P = 0.002$). Visceral and hepatic fat did not necessarily influence the degree of FMD. Thus, moderate intensity exercise can improve the endothelial function of conduit arteries regardless of improvement in hepatic or visceral adiposity.

Exercise regimens have also been rigorously evaluated in diabetic patients with NAFLD. In the literature, there is a similar dilemma regarding exercise and diabetic patients, as it is unclear which form of exercise provides the most benefit for glycemic control and improving cardiovascular fitness[43]. Bacchi *et al*[43] examined how a combination of aerobic and resistance exercise impacts insulin sensitivity and adiposity of the liver, viscera, and midsection in patients with both diabetes and NAFLD. This randomized control trial was derived from a trial which that compared the metabolic effects of supervised resistance *vs* aerobic exercise for type 2 diabetics with sedentary lifestyles. The aerobic arm participated in 60-min sessions on various cardio machines, exercising at a goal of 60%-65% of their heart rate reserve. The resistance arm participated in weight lifting exercises with 3 series of 10 repetitions. Both groups exercised thrice weekly for four months and maintained their old diets. There was a significant reduction in hepatic adiposity in both groups, and nearly 25% of patients in both groups no longer had hepatic steatosis at the end of the trial. Insulin sensitivity, glycosylated hemoglobin, and high density lipoprotein serum levels were reduced similarly in both groups, as was abdominal and visceral adiposity. Thus, among diabetics with NAFLD, resistance and aerobic exercise both result in a reduction in hepatic steatosis and one form of exercise is not superior to another.

The benefit of exercise in NAFLD patients is undeniable and extends well beyond the liver by improving

metabolic derangements such as insulin resistance and atherosclerotic disease and facilitating the development of cardiorespiratory fitness, among others. Since Keating's comprehensive systematic review in 2012 on the effects of exercise on NAFLD, more data has surfaced in support of exercise and lifestyle behavior modification, and recent studies have attempted to explore and define the potential benefits of exercise on both the liver and cardiovascular system[13]. However, the ability of researchers to definitively suggest specific interventions for the treatment of NAFLD is limited by numerous factors. It is still unclear exactly which form of exercise is superior, how long the exercise sessions should be, or if weight loss is required in the treatment of the disease. Studies which examine these questions are limited by the small number of patients studied. Very few studies examine a single intervention in isolation, as it is difficult to control for diet or exercise alone.

Additionally, it is challenging to determine exactly what aspect of an intervention led to the improvement in hepatic steatosis or metabolic derangements. Researchers, for the most part, rely on imaging or serology to determine the effects of their interventions, but these modalities are imperfect in evaluating the change or degree of steatosis. Biopsy, the gold standard in diagnosing NASH, is also difficult to obtain due to cost as well as health risks to the patient. Perhaps more studies should be performed in the population of living liver donors, since these patients require biopsy for donor evaluation, and the benefits of biopsy for both the future recipient and the donor likely outweigh the risks of biopsy. Many unknowns remain, and hopefully more research within this field will help in the creation of more evidence-based guidelines for physical activity as a treatment of NAFLD.

From the studies selected for this review, it seems that recommending moderate intensity exercise, which incorporates both aerobic and resistance components, is reasonable to treat NAFLD in able-bodied patients. While not discussed in depth in this review, encouraging healthy eating may offer additional health benefits for these patients. However, there undoubtedly is wide variation on the degree of functional mobility and ability for these patients to adhere to such a treatment plan. Physicians should also consider aspects which may limit a person's ability to participate in lifestyle modification such as motivation, access to gym facilities and healthy food, and physical limitations. Studies of diabetic patients indicate that as the level of intensity of an exercise program increases, motivation and adherence diminish[43]. As the rates of obesity and morbid obesity continue to rise, a larger proportion of the NAFLD patient population will present challenges for treatment to physicians throughout the country. Acceleration and hybrid training, along with other low impact exercises may provide modest benefit for those patients limited by their body habitus or their poor cardiorespiratory fitness. Tackling this growing epidemic will likely require a strong multidisciplinary approach, combining physical activity, nutrition, and behavioral modification to develop a solution for this diverse patient population.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is becoming more prevalent throughout the world and increasingly problematic in terms of costs to the healthcare system and individual. While NAFLD carries with it the risk of progression to worsening hepatic steatosis and cirrhosis, the presence of NAFLD, a disease highly associated with the metabolic syndrome, also increases the risk of cardiovascular morbidity and mortality. The first line treatment for NAFLD incorporates both dietary modifications and exercise. However, there is a paucity of high-powered studies and substantial evidence to support this treatment as well as to prove the impact of this treatment on both the metabolic and cardiovascular derangements seen in NAFLD. The goal of this review is to examine the available evidence for exercise in NAFLD from 2010-2015 and to determine the efficacy of exercise to treat NAFLD and its concurrent disease states. The secondary aim of this review was to determine if any form of exercise or particular length or duration of exercise was more efficacious in treating this disease state and improving factors such as insulin sensitivity, hepatic steatosis, and visceral adiposity.

Research frontiers

Numerous studies examine the effect of employing either resistance or aerobic exercise in the treatment of NAFLD. Researchers proposed modest exercise routines which can reasonably be completed by a substantial proportion of the NAFLD population. However, these programs may be difficult for patients with morbid obesity, advanced age, and other severe physical limitations. Newer studies have examined the benefit of low impact exercise, such as acceleration and hybrid training, in the treatment of NAFLD. While the results are modest, programs such as these may be employed to allow physically limited patients to achieve improvements in functional mobility as well as improvements in cardiovascular and metabolic health.

Innovations and breakthroughs

Both resistance and cardiovascular exercise regimens have been shown to demonstrate benefit in treating NAFLD. Newer forms of exercise, such as acceleration training and hybrid training, seem promising as well. Additionally, decreasing one's sedentary time through increased physical activity even if the activity is low intensity may have health benefits for this patient population.

Applications

The studies reviewed support the benefits of lifestyle intervention on NAFLD and its resultant cardiovascular and metabolic disease. It is reasonable to recommend moderate intensity exercise which comprises of both aerobic and resistance exercise for patients.

Terminology

NAFLD incorporates a range of disease from simple hepatic steatosis no nonalcoholic steatohepatitis. NAFLD is closely associated with the metabolic syndrome, and recent research has identified a strong association with cardiovascular disease, which represents the largest cause of mortality for patients with NAFLD.

Peer-review

In this invited manuscript, the authors aimed to conduct a systematic review evaluating published literature to assess the efficacy of exercise as a treatment for NAFLD and its effect on insulin resistance, dyslipidemia and amount of visceral adiposity. It seems to the authors a good manuscript, correctly developed with a suitable order, creates an awareness on the subject.

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