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REVIEW

Recent advances in non-invasive magnetic resonance imaging assessment of hepatocellular carcinoma

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Abstract

Magnetic resonance (MR) imaging of the liver is an important tool for the detection and characterization of focal liver lesions and for assessment of diffuse liver disease, having several intrinsic characteristics, represented by high soft tissue contrast, avoidance of ionizing radiation or iodinated contrast media, and more recently, by application of several functional imaging techniques (*i.e.*, diffusion-weighted sequences, hepatobiliary contrast agents, perfusion imaging, magnetic resonance (MR)-elastography, and radiomics analysis). MR functional imaging techniques are extensively used both in routine practice and in the field of clinical and pre-clinical research because, through a qualitative rather than quantitative approach, they can offer valuable information about tumor tissue and tissue architecture, cellular biomarkers related to the hepatocellular functions, or tissue vascularization profiles related to tumor and tissue biology. This kind of approach offers *in vivo* physiological parameters, capable of evaluating physiological and pathological modifications of tissues, by the analysis of quantitative data that could be used in tumor detection, characterization, treatment

selection, and follow-up, in addition to those obtained from standard morphological imaging. In this review we provide an overview of recent advanced techniques in MR for the diagnosis and staging of hepatocellular carcinoma, and their role in the assessment of response treatment evaluation.

Key words: Liver; Cirrhosis; Hepatocellular carcinoma; Magnetic resonance; Transarterial chemoembolization; Contrast media

Core tip: Magnetic resonance (MR) of the liver is an important diagnostic option for detection and characterization of focal liver lesions. To date, beside the standard morphological sequences, new functional imaging tools (*i.e.*, diffusion-weighted sequences, hepatobiliary contrast agents, perfusion imaging, MR-elastography, or radiomics analysis) have been introduced in clinical practice. The aim of functional imaging is to provide *in vivo* quantitative complementary functional data related to the tissue or tumor modifications, offering useful comprehensive information about the biology, behavior, and prognosis of hepatocellular carcinoma lesions. This functional approach may help clinicians correctly manage cirrhotic patients, also after therapeutic treatment.

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INTRODUCTION

Liver cancer is the fifth most frequently diagnosed malignancy among men and the ninth among women. Recently, it has risen from the third to the second cause of death from cancer, accounting for nearly 746000 deaths in 2012. In some regions, like Eastern and South-Eastern Asia, mortality almost equals incidence with an overall ratio of 0.95[1]. The most common histological subtype of liver cancer is hepatocellular carcinoma (HCC), representing more than 90% of cases. The incidence of HCC increases with advanced age, reaching, at least in developed countries, a peak at 70 years[2]. In up to 90% of cases, HCC occurs in the setting of liver cirrhosis and overall, one-third of cirrhotic patients will develop HCC during their lifetimes[3].

The primary risk factor for HCC is still represented by chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection[4], with a prevalence of virus B infection in Eastern countries and a prevalence of virus C infection in Western countries. Other causes of cirrhosis comprise alcohol abuse, non-alcoholic fatty liver disease (NAFLD), and less frequent disorders such as hemochromatosis. All etiologies could lead to cirrhosis and may be complicated by tumor formation, but the risk is higher in patients with hepatitis infection. In the coming years, the diffusion of new antiviral agents for HCV[5], vaccination and therapy for HBV[6], and prevention campaigns are expected to reduce the burden of chronic viral liver disease and its complications, including HCC[7]. On the other hand, the widespread epidemic of obesity is expected to induce a significant increase in the incidence of NAFLD and its complications, such as NASH, cirrhosis, and HCC[8,9].

Liver cirrhosis is a common underlying condition associated with hepatocarcinogenesis. Cirrhosis develops after a long period of chronic liver disease, when the risk of HCC is still low. The nodules that could be potentially found in a cirrhotic liver comprise: Small and large regenerative nodule (RN), low-grade dysplastic nodule (LGDN), high-grade dysplastic nodule (HGDN), early HCC, well-differentiated HCC, and moderately-poorly differentiated HCC. Hepatocarcinogenesis is a multistep event during which cell density increases, Kuppfer cells decrease, nodules enlarge, and hemodynamics change. In the initial phase, normal arterial supply decreases but portal perfusion is still present. Later, intranodular arterial vascularity increases due to the appearance of unpaired arteries (capillarization) while portal blood supplies progressively decrease[10]. Simultaneously, organic anionic transporting polypeptide (OATP), transporters of bile salts, gradually decrease. OATP expression levels are high in RNs and LGDNs and lower in many HGDNs, early HCCs, and progressed HCCs. The hemodynamic changes are well depicted during dynamic multidetector computed tomography (MDCT) and magnetic resonance imaging (MRI), and both European and American guidelines have endorsed this technique for the diagnosis of HCC > 1 cm, based on the typical hallmarks of hypervascularity in arterial phase with wash-out in portal phase, thereby avoiding liver biopsy[11,12].

However, there remains a high rate of false negative, ranging from 25%-30%, in particular for nodules < 2 cm[13,14], which actually are the most often encountered focal liver lesions, thanks to the widespread of surveillance programs. In these small nodules, hemodynamic changes of hepatocarcinogenesis are in an early stage, since neoangiogenesis is incomplete and they are still mainly filled by portal vessels, in contrast to progressed HCC. MRI in part overcomes these limits. It has been recently demonstrated that this diagnostic technique has a higher diagnostic performance over computed tomography (CT) in the detection of high-risk nodules[15]. This is due to its high contrast resolution and to its multiparametric characteristics. In fact, it is known that hyperintensity on T2 weighted sequences and restricted diffusion in diffuse weighted images (DWI) are features of malignancy[16]. Moreover the recent introduction of hepatospecific MRI contrast agent

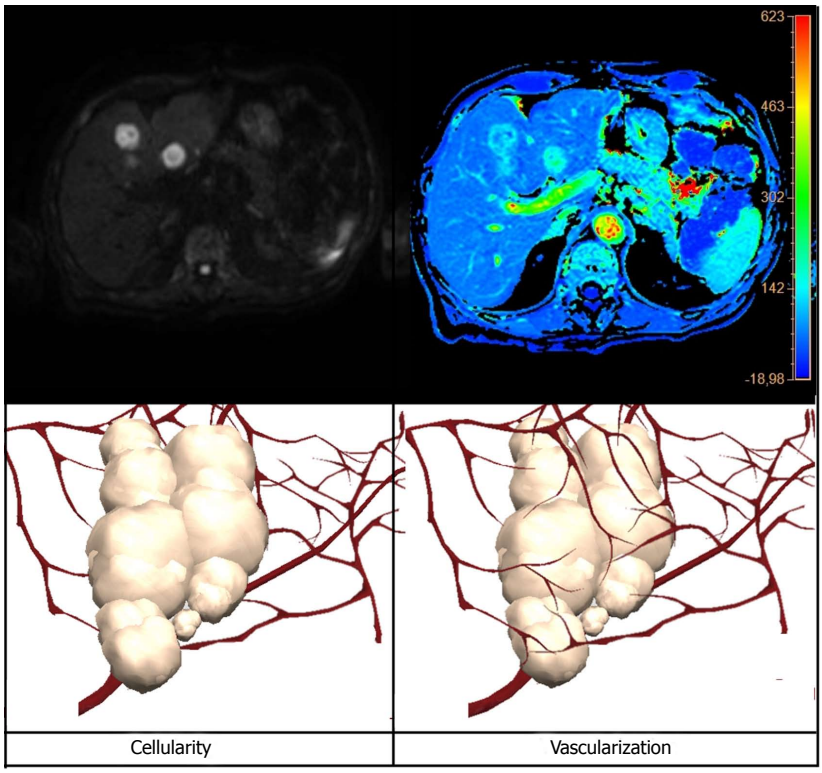

Figure 1 Schematic comparison between diffusion weighted images (on the left) and perfusion maps (on the right) showing the meaning from the pathophysiological point of view of the two different functional magnetic resonance techniques. The diffusion offers qualitative information strictly related to tissue cellularity, while perfusion sequences offer qualitative information about tissue vascularization.

gadolinium-ethoxybenzyl-dieth-ylenetriamine pentaacetic acid (Gd-EOB-DTPA, Primovist®; Bayer Schering Pharma, Berlin, Germany), which gives information not only on vascular changes but also on hepatocyte function, raises the sensitivity for the detection of early HCC to 91%-93%[17]. Based on this feature, Kim BR and colleagues[16] demonstrated that readers had significantly higher detection sensitivity for early HCCs with MRI than with multidetector CT (78.6% *vs* 52.4%, $P = 0.001$; 71.4% *vs* 50.0%, $P = 0.011$; and 73.8% *vs* 50.0%, $P = 0.001$, respectively), as shown by 30 more LI-RADS category 4 early HCCs identified at MRI.

The correct characterization of all nodules possibly encountered in a cirrhotic liver is of paramount importance because they are managed completely differently. In fact, while regenerative and dysplastic nodules deserve a strict follow-up, HCC should be treated with the more suitable therapeutic option, according its stage. This is clearly defined by the Barcelona Clinic Liver Cancer (BCLC) staging system, adopted in all Western countries and endorsed by both American and European guidelines.

In this context, beside traditional radiological techniques, new functional imaging tools have been introduced in clinical practice in order to provide not only morphological information but also functional data information. Functional magnetic resonance imaging encompasses a wide range of advanced techniques capable of evaluating physiological and pathological modifications of tissues, by the analysis of quantitative data, in addition to those obtained from standard morphological imaging. These techniques may include diffusion-weighted sequences, hepatobiliary contrast agents, perfusion imaging, MR-elastography, and more recently radiomics analysis. In particular, perfusion imaging (related to vascular profile) and diffusion imaging (related to cellular profile) (Figure 1) techniques have been extensively studied during various steps of HCC evolution, from initial assessment of vascular modifications in cirrhotic liver, through its progression in tumor lesion, and finally to its follow-up after treatment. Hence, in this review, we provide an overview of recent advances and techniques in MR studies for the diagnosis and the staging of HCC.

CONTRAST MEDIA: EXTRACELLULAR AND HEPATOBILIARY AGENTS

Gadolinium is a paramagnetic ion that shortens T1 relaxation time in tissues and, therefore, produces an increase in signal intensity[18]. Based on bio-distribution, there are three categories of gadolinium-based contrast agents: extracellular fluid agents (ECFAs), blood pool agents (BPCAs), and targeted and organ-specific contrast agents, such as hepatocyte-specific contrast agents (HCAs). ECFAs and HCAs are the most commonly used in liver imaging. ECFAs consist of gadolinium chelated to an organic compound, such as DTPA[19]. They are

Table 1 Gadolinium-based magnetic resonance imaging contrast agent

Contrast agent	Category	Relaxivity	Structure	Concentration (mmol/mL)	Reccomended dosage (mmol/kg)
Gadoterate-meglumine	ECFAs	Standard	macrocyclic	0.5	0.1
Gadobutrol	ECFAs	Standard	macrocyclic	1.0	0.1
Gadoteridol	ECFAs	Standard	macrocyclic	0.5	0.1
Gadopentetate- dimeglumine	ECFAs	Standard	Linear	0.5	0.1
Gadoversetamide	ECFAs	Standard	Linear	0.5	0.1
Gadodiamide	ECFAs	Standard	Linear	0.5	0.1
Gadofosfaset-trisodium	BPCAs	High	Linear	0.25	0.03
Gadobenate- dimeglumine	HCAs	High	Linear	0.5	0.1
Gadoxetate-disodium	HCAs	High	Linear	0.25	0.025

ECFAs: Extracellular fluid agents; BPCAs: Blood pool agents; HCAs: Hepatocyte-specific contrast agents.

further divided in standard relaxivity macrocyclic agents, standard relaxivity linear agents, and high relativity linear agents (Table 1). The details regarding the advantages and disadvantages of each contrast category is beyond the scope of this article, but in general, there is little clinical difference[20]. The standard dose is 0.1 mmol/kg typically injected intravenously at a rate of 2 mL/s followed by a normal saline "flush" of 20 to 50 mL. After the injection, ECFAs are rapidly cleared from the intravascular space through the capillaries into the extracellular space. They are mainly eliminated by renal excretion and have imaging dynamics comparable to the extracellular iodinated contrast media used in CT. However, MRI is more sensitive to the effects of gadolinium than CT is to the effects of iodine, because gadolinium has an amplification effect due to the number of adjacent water protons relaxed by a single gadolinium atom[19,21]. In summary, ECFAs enter into the liver through the hepatic artery and portal vein and are freely redistributed into the interstitial space; they demonstrate vascular perfusion by distributing and allow the evaluation of liver lesions based on assessment of vascularity. The combination of arterial phase hyperenhancement followed by washout appearance in the portal venous and/or delayed phase is the key diagnostic feature of HCC[11,12] (Figure 2).

The pathophysiologic basis for arterial phase hyperenhancement in HCC is related to the increasing of the intranodular arterial supply during hepatocarcinogenesis[22]. The mechanisms underlying washout appearance in HCC depend on a range of factors: early venous drainage of contrast material from the tumor, progressive enhancement of background liver, reduced intranodular portal venous blood supply, tumor hypercellularity with corresponding reduction in extracellular volume, and intrinsic hypoattenuation/hypointensity[23]. In cirrhotic patients, this enhancement pattern has approximately 100% specificity for lesions larger than 2 cm and approximately 90% specificity for those of 1-2 cm[24]. However, the main limitation with ECFAs for diagnosis and staging HCCs is low per-lesion sensitivity, because an atypical vascular behavior is quite common in small (< 2 cm) nodules and approximately one-third of these are malignant ("the one-third rule")[25]. Indeed, the intranodular hemodynamic changes during carcinogenesis start with an arterial hypovascularity with portal perfusion still present, followed by a decrease of both arterial and portal blood supply, then followed by an increase in arterial vascularity to isovascular, and, finally, to a hypervascular pattern[12].

On the other hand, several recent studies demonstrated that the expression of OATP diminishes during hepatocarcinogenesis[26]. Moreover, OATP 8 expression level decreases prior to complete neoangiogenesis, with elevation of arterial flow and reduction of portal venous flow[27]. Thanks to their lipophilic characteristics, HCAs, after the intravascular/interstitial distribution, are taken up by functioning hepatocytes, metabolized, and excreted into the bile through the OATP 8: Consequently, nodules with low or no OATP expression (the majority of HCC, many early HCCs, and some high-grade dysplastic nodules) do not uptake HCAs and appear hypointense in the hepatobiliary phase (HBP) (Figure 2). A recent meta-analysis has shown that the impact of HBP on a per-lesion sensitivity is significant, in particular the use of Gd-EOB-DTPA allowed a sensitivity of 87% *vs* 74% (P = 0.03) the one without HBP[28]. Based on these considerations, the current contrast agents applied in the study of the liver are the gadobenate dimeglumine (Gd-BOPTA/Dimeg, MultiHance®, Bracco, Milan, Italy), which is a chelate of the paramagnetic gadolinium ion salified with two molecules of meglumine, and gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid (Gd-EOB-DTPA, Primovist®, Bayer Schering Pharma, Berlin, Germany), which is a highly water-soluble contrast agent with an ethobenzyl group attached to gadolinium diethylenetriamine pentaacetic acid[29]. The approved dose of Gd-BOPTA for hepatic imaging is 0.05 mmol/kg (0.1 mL/kg of a 0.5 mol/L solution)[30], and it should be administered undiluted followed by a normal saline "flush" of 20 to 50 mL. Hepatic uptake represents 2%-4% of the injected dose for Gd-BOPTA, and the HBP is typically performed between 45 and 120 min after injection and is necessary in order to achieve sufficient enhancement.

The approved dose of Gd-EOB-DTPA is 0.025 mmol/kg, which is considered the minimum effective dose for the detection of liver lesions in the hepatobiliary phase.

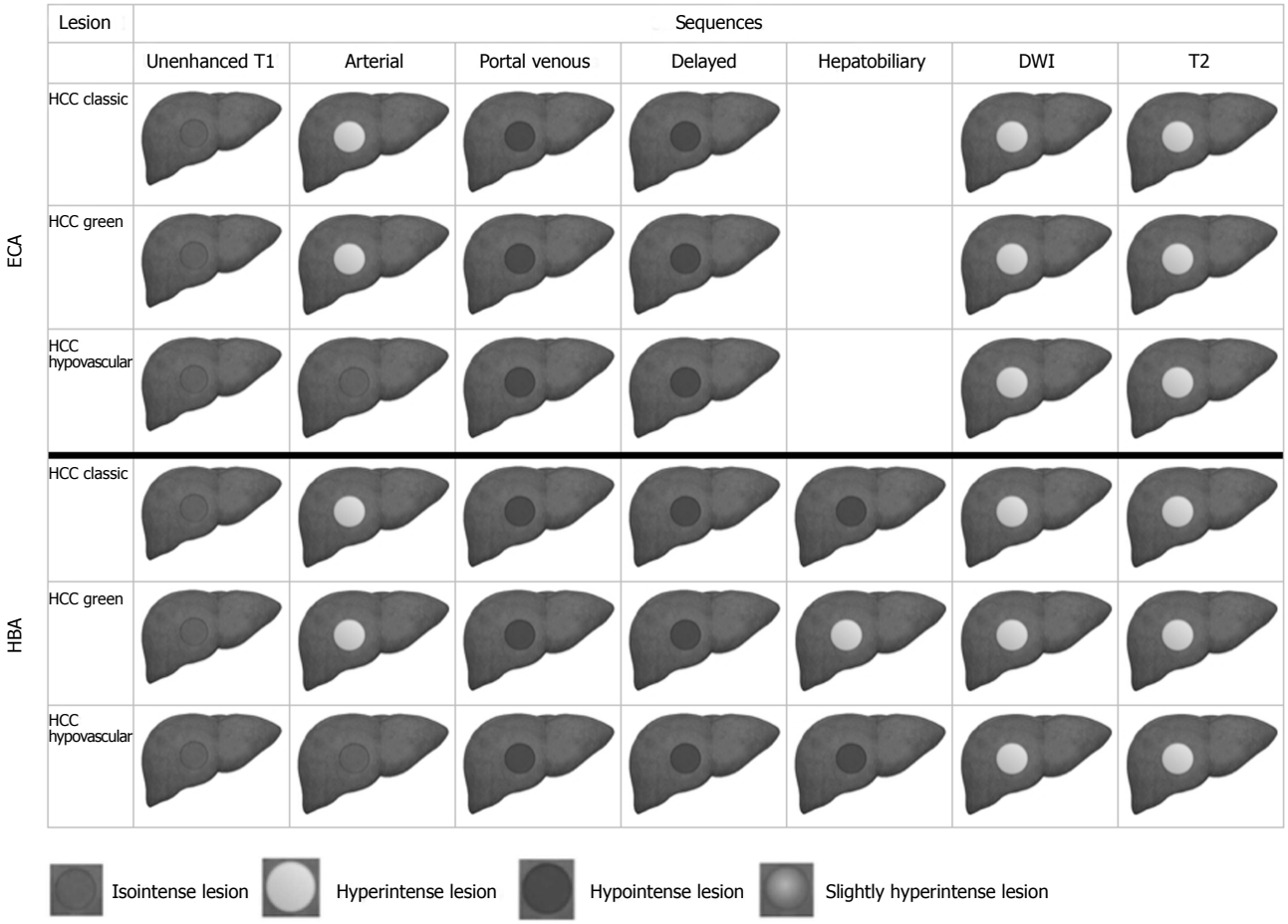

Figure 2 Schematic representation showing dynamic contrast enhanced sequences, diffusion weighted images, and T2-weighted features in typical, green, and hypovascular hepatocellular carcinoma, comparing information from extracellular contrast agent and hepatobiliary contrast agent. ECA: Extracellular contrast agent; HBA: Hepatobiliary contrast agent; DWI: Diffusion weighted images.

The modalities of gadoxetic acid administration were addressed in the ESGAR consensus statement[31] (flow-rate of 1-2 mL/s followed by a 20 mL saline flush at 1-2 mL/s using a bolus triggering technique). Hepatic uptake represents 50% for Gd-EOB-DTPA, and the HBP reaches its maximum intensity approximately 20 min after injection with gadoxetate disodium and persists for several hours[32]. The clinical use of liver-specific contrast agents allows the radiologist to obtain morphologic and vascular-related information, although an overlap between delayed phase and hepatocyte phase have to be considered during dynamic evaluation[33]. A recent meta-analysis[34] reported that in trials of MRI that directly compared test performance using different contrast agents, use of HCAs was associated with higher sensitivity than ECAs (difference of 13%), with no difference in specificity. The difference was somewhat greater for HCC lesions smaller than 2 cm (difference of 15%). These finding were stressed by the ESGAR consensus[31], who stated that a Gd-EOB-DTPA MR examination should be performed in order to characterize an undetermined focal liver lesion of 10 mm or larger in the cirrhotic liver. In summary, HCAs allow a comprehensive non-invasive imaging assessment of the liver parenchyma, intrahepatic lesions depiction or characterization, hepatic vasculature, and the biliary tree in a single examination. They have several advantages in the evaluation of the cirrhotic liver including: (1) Higher sensitivity for the diagnosis of HCC, in particular for lesions smaller than 2 cm[34]; (2) improved characterization of arterially enhancing lesions without definite washout on subsequent imaging[35]; (3) the possibility to differentiate arterially enhancing lesion *vs* pseudolesions[36]; and (4) detection of lesions with decreased uptake evidenced only in the HBP that are likely to be precancerous or borderline lesion[37].

PERFUSION IMAGING

Perfusion MRI in the assessment of HCC focuses on the detection and characterization of lesions[38-40], the evaluation of response to therapy[38,41-44], and determination of prognosis[44,45] (Table 2).

The basis of dynamic contrast-enhanced (DCE)-perfusion MR imaging is the acquisition of multiple image sets, every few seconds, through the tumor or as much of the organ as possible, after gadolinium injection. The rate and pattern of contrast enhancement reflects

Table 2 Magnetic resonance imaging perfusion with dynamic contrast-enhanced magnetic resonance imaging in the assessment of hepatocellular carcinoma, focus on diagnosis, characterization, response to therapy, and prognosis

Ref.	Year	Magnet (Tesla)	Contrast agent	Parameters
Diagnosis and characterization				
Taouli *et al*[38]	2013	1.5 T	Gadobenate-dimeglumine and gadopentetate-dimeglumine	AF, VF, ART, DV, MTT
Chen *et al*[39]	2017	3 T	GD-EOB-DTPA	Ktrans, Kep, iAUC, max-Ktrans
Jajamovich *et al*[40]	2016	3 T	Gadobenate-dimeglumine	ART, K trans, ve, kep, τ
Abdullah *et al*[61]	2008	1.5 T	Gadoterate-dimeglumine	HPI, MTT, DV, TF, AF, PF
Response to therapy				
Ippolito *et al*[41]	2016	1.5 T	GD-EOB-DTPA	ME, MRE, RAE, RE, RLE, RVE, TTP
Taouli *et al*[38]	2013	1.5 T	Gadobenate-dimeglumine and Gadopentetate-dimeglumine	AF, VF, ART, DV, MTT
Chen *et al*[45]	2016	1.5 T	Gadodiamide	Peak, Slope, AUC, Ktrans, Kep, Ve
Prognosis				
Chen *et al*[45]	2016	1.5 T	Gadodiamide	Peak, Slope, AUC, Ktrans, Kep, Ve
Chen *et al*[45]	2016	1.5 T	Gadodiamide	ART, AF, PF, TF, MTT, DV, PEAK, SLOPE, AUC

ART: Arterial fraction; K trans: Contrast agent transfer rate constant from plasma to extravascular extracellular space; VE: Extravascular extracellular volume fraction; Kep: Contrast agent intravasation rate constant; τ: Mean intracellular water molecule lifetime; ME: Maximum enhancement; MRE: Maximum relative enhancement; RAE: Relative arterial enhancement; RE: Relative enhancement; RLE: Relative late enhancement; RVE: Relative venous enhancement; TTP: Time to peak; HPI: Hepatic perfusion index; MTT: Mean transit time; DV: Distribution volume; TF: Total blood flow; AF: Arterial blood flow; PF: Portal blood flow; AUC: Area under the gadolinium distribution-time curve.

the time evolution of the contrast agent within the tissue, which occurs as a result of the microcirculatory pathophysiological changes. Perfusion MRI could extend the currently used qualitative assessment applied for the differential diagnosis of lesions, by applying quantitative metrics to describe their vascular behaviour.

The main purpose of MRI perfusion is the quantification of vascular characteristics of HCC, because the growth and progression of histological malignancy of HCC are associated with new blood vessels formation[46] (angiogenesis). Moreover, the targets of anti-angiogenic drugs, recently used for HCC treatment, are represented by these new blood vessels and, therefore, the perfusion, as a functional imaging technique, may be suitable for evaluating patients treated with these agents[47-50].

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) provides non-invasive imaging biomarkers that can measure changes in tumor blood flow, vascular permeability, and interstitial and intravascular volumes[40,43,47] and can predict the survival outcome in patients with HCC[51-53]. Generally, DCE-MRI consists of acquisition of T1-weighted MR images before, during, and after intravenous injection of a gadolinium-based contrast agent[40]. The contrast agent extravasates at level of tumor tissue, from intravascular to the extravascular extracellular space (EES) with increased T1-w signal[43,54,55]. This extravasation to EES in the tumor tissue depends on vessel leakiness (permeability) and blood flow (perfusion), and so the signal measured with DCE-MRI could be sensitive to alterations in vascular permeability, EES, and blood flow[43,54].

DCE-MRI signals can be quantified using a semi-quantitative (model free) or quantitative (model based) analysis[56]. Both analysis methods have several parameters related with tumor angiogenesis[54,57] and can give different information on liver and tumor perfusion[56]. Briefly, with the semi-quantitative analysis, all perfusion parameters are extracted directly from time-signal intensity (SI) curves [*e.g.*, AUC, maximum SI or peak enhancement ratio, wash-in slope, mean transit time (MTT)], derived from different dynamic contrastographic sequences. Although widely used, semi-quantitative analysis is highly affected by the acquisition systems and comparison and quantification of these parameters can be difficult[56,57] because the true concentration of contrast agent in the tissues is not estimated (Figure 3).

Quantitative analysis depends on fitting the time SI curves with the changes in concentration of the contrast agent using pharmacokinetic techniques using several kinetics models based on different physiological assumptions made[56]. These kinetics models can be bi-compartmental models (taking into account vessels and EES) or mono-compartmental (taking into account the vascular space because of the typical architecture of the liver)[56], with a double or single input system (arterial and portal or arterial alone), conventional compartment (CC) models vs distributed parameters (DP) models[54,56].

Several parameters extracted with quantitative analysis are related to the influx of contrast agent from the intravascular space to the EES (K trans) and its reverse (Kep), the volume fraction of EES (Ve), which is an indirect expression of the cellular density of the tissue[43,54,56].

In comparison to the semi-quantitative analysis, these parameters are more time consuming because they generate parametric maps through a pixel-by-pixel curve fitting process. Although the histogram analysis and the heterogeneity of these parametric maps are more computationally demanding, they may also provide additional information[43,56]. Moreover, numerous pharmacokinetic models have been proposed by Tofts

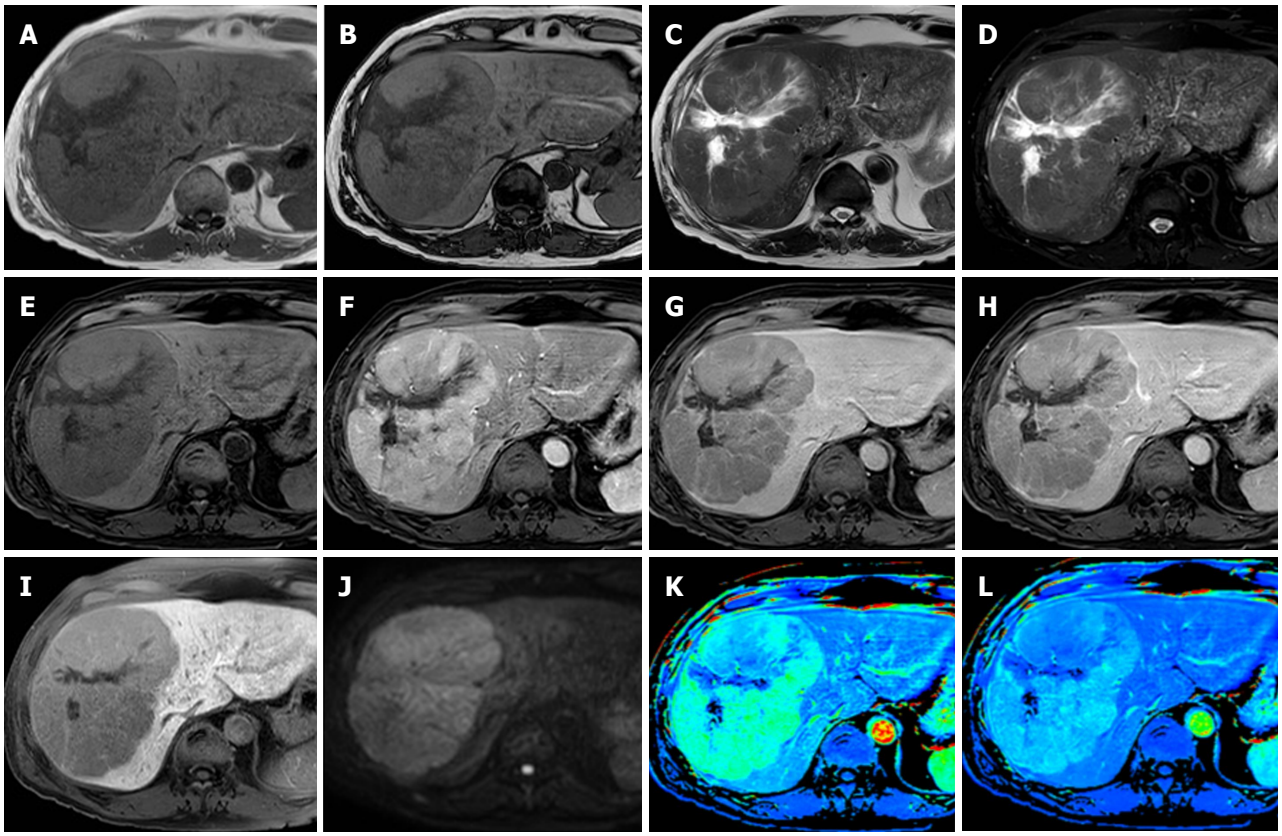

Figure 3 Gd-EOB-DTPA enhanced magnetic resonance images of a 67-year-old male patient with large hepatocellular carcinoma lesion in the right liver lobe. Panels A-B: T1-weighted sequences "in and out of phase" demonstrate a heterogeneous mass slightly hypointense without a signal drop in "out of phase" sequence. Panels C-D: T2-weighted image without and with fat saturation demonstrates a slightly hyperintense mass with a central, homogeneous hyperintense area, as per necrosis. Panels E-H: Dynamic contrast-enhanced images delineate the typical contrast behavior of hepatocellular carcinoma (HCC): Hyperenhancement during the arterial phase (F) followed by wash-out in portal and delayed phase (G-H). In the hepatobiliary phase image 20 min after Gd-EOB-DTPA injection the nodule appears highly hypointense compared with the surrounding enhanced liver (panel I). Panel J: On the diffusion weighted image, HCC lesion is hyperintense due to the restriction of water diffusion. Panel K-L: Perfusion images derived from semiquantitative analysis (relative arterial enhancement and maximum enhancement) the HCC is characterized by high vascularity intensity signals, shown as hot-spots signals.

et al[58], Brix *et al*[59], and Larsson *et al*[60], using a single arterial input function[43]. Because HCC receives major blood supply from hepatic neo-arteries and often arise from a cirrhotic liver, the single input model (considering only the arterial input) and the dual compartment model (because of the alteration in the EES) are frequently both used in the literature[54,56].

However, because of numerous DCE-MRI–related limitations, parameters derived from these pharmacokinetic models may lack sufficient precision for clinical application[38], and there is no consensus regarding the pharmacokinetic model that should be used to quantify HCC perfusion parameters, even if some studies demonstrated that some pharmacokinetic models can be equivalent in the results[40].

All these possibilities and differences in the field of DCE-MRI led to literature studies with different results.

In general, two recent studies demonstrated that HCC had significantly higher peak, slope, AUC, arterial fraction, and arterial flow but lower portal flow, distribution volume, and MTT than the liver[45]. HCCs with high peak are correlated to a longer overall survival (OS) in comparison with HCC with low peak[45] before systemic therapy. Secondly, high peak reduction assessed early (1 wk) after systemic therapy can be related to OS[44].

DCE-MRI semi-quantitative parameters (relative arterial, venous, and late enhancement; maximum enhancement; maximum relative enhancement, and time to peak) potentially can be used also to differentiate residual viable tumor tissue and effective treated lesions after TACE or RFA[41]. Some of them, in a multivariate analysis, seemed also to predict the response to radiotherapy RT[42].

Some groups found that with DCE-MRI it is possible to quantify the perfusion in the liver and HCC with an increased arterial flow and decreased portal venous flow in HCC compared with cirrhotic liver, with significant differences in the degree of arterial versus portal venous blood flow in treated and untreated HCCs[38].

Perfusion parameters could be correlated to the grading differentiation of HCC, but in most of the cases, there were no significant differences in perfusions and grade of HCC differentiation, with the exception of the arterial fraction (ART)[40]. The ART parameter is a value estimated each time through perfusion equations

obtained from the addition of the two input inflow (arterial and portal) into one[40]. Moreover, it has been suggested that ART can be used to assess response to local regional therapy in HCC[38,40,61].

In a recent study from Chen *et al*[39], the max-Ktrans seemed to correlate with tumor grades (rho = -0.382, $P = 0.028$). The Ktrans, Kep, and iAUC of high-grades HCC were significantly lower than that of low-grades HCC ($P = 0.001$, 0.031, 0.003, respectively), but there was no statistically significant differences for Ve between high grade and low grade HCC ($P > 0.05$)[39].

These results suggest that DCE-MRI can be useful as a non-invasive marker of HCC angiogenesis, but new equipment and sequences and models are still under investigation. New equipment will be applied in the near future to quantify the perfusion of HCC, as a biomarker of degree of malignancy, prognosis, and response to therapy[38-40].

DIFFUSION WEIGHTED IMAGING

DWI is a functional MRI sequence that allow the characterization of biological tissues based on the diffusion properties of water molecules, providing information about tissue cellularity and about the integrity of cellular membranes[2]. In fact, in high cellular tissue, the higher density of hydrophobic cellular membranes reduces the "apparent" diffusion of water protons[62], thus the water diffusion can be considered relatively "restricted". More simply the "diffusion restriction" refers to a tumor signal intensity that is higher than the surrounding parenchyma (the liver for example) on high b-value DW MR images, and, to date, DW-imaging represents an integral part of the routine MR protocol for liver disease (Figure 4).

In 2010, Taouli *et al*[63] defined DW MR imaging, an attractive technique, which was reaching a potential for clinical use in the abdomen, particularly in the liver. Less than a decade later, all the potential uses of DWI are greatly shown, and diffusion can be considered a useful tool for the diagnosis of focal liver lesions, with better results than T2-weighted images[64] especially in HCC[65]. There are various reasons why: DWI adds useful qualitative and quantitative information to standard sequences; it has a short acquisition time and can be easily included to existing protocols; and it does not need the use of contrast materials[66,67].

Although several DW imaging sequences can be applied to evaluate the liver, the single shot spin-echo (SE) echo-planar technique is the most frequently used in combination with fat suppression. Recent studies[68] compared free breathing (FB) *vs* respiratory triggered (RT) DWI for detecting HCC, using a 3 T scanner, a 32-channel torso-cardiac phased-array coil, and dual-source parallel radiofrequency excitation and transmission technology. They concluded that FB-DWI provided better image quality and showed higher detectability of HCCs in patients with chronic liver disease compared to RT-DWI, without significantly reducing the SNR of the normal liver parenchyma or the lesion-to-non lesion CNR. DW imaging should not be considered a stand-alone sequence, but should be integrated in MR protocols: The combination of Gd-EOB-DTPA and DWI could allow the assessment of the three main processes in the hepatic multistep carcinogenesis (vascular changes, hepatocyte change, and tissue diffusivity). A recent meta-analysis showed that the combination of gadoxetic acid-enhanced MRI and DWI significantly improved both diagnostic accuracy and specificity for HCCs associated with chronic liver disease[69]. Several studies underline the importance that DWI adds to dynamic contrast-enhanced MRI, in characterization of small or atypically enhancing lesions[70,71]. In particular, Briani *et al*[71] demonstrated that the hypovascular lesions ≥ 10 mm that appeared hyperintense in DWI are associated with progression to hypervascular HCC. DWI can not only indicate the morphological characteristics of a lesion with a qualitative assessment, but with apparent diffusion coefficient (ADC) measurement, can also provide a quantitative index of diffusion characteristics, analyzing structure and tissue components. Some authors[70,72] suggested that a lesion-to-liver ADC ratio cut-off value of 0.92 may offer good sensitivity, specificity, and accuracy in differentiating HCC vs dysplastic nodules (DN). Inchingolo *et al*[70], furthermore, obtained higher values (sensitivity 90.91%, specificity 80.95%, and accuracy of 83.55%, when the group of LGDNs was compared to the group that included both HGDNs and HCCs, with a cut-off of 0.95. Jiang *et al*[73] conducted a retrospective analysis of the correlation between qualitative and quantitative DWI and HCC tumor grade. They found that while SI values on DWI could distinguish only between well-differentiated HCC and moderately or poorly differentiated HCC, ADC values could distinguish between well, moderately, and poorly differentiated HCC, with the consequence of a better pre-operative and non-invasive histological characterization. Further applications of DW imaging are still ongoing, and larger studies are needed to validate these results. One example is the application of DWI concerning the prediction of microvascular invasion (MVI) in HCC. MVI still remains one of the important prognostic factors of HCC recurrence, especially after surgical resection or liver transplantation[74,75]. In the past, other imaging characteristics have previously been suggested as predictors of MVI, such as tumor size, shape and margin, capsule, peritumoral enhancement, and dynamic enhancement pattern; but recently Yang *et al*[76] proposed a new integrated evaluation of T2 and DWI images by defining the concept of "diffusion- and T2-weighted imaging mismatch". They demonstrated that this new "DWI/T2 mismatch" was an independent predictor of MVI (odds ratio 4.521, $P = 0.035$), with a high specificity (95.65%). Another potential application of DWI is the assessment of liver tumor response to novel therapy. In fact, while a change in tumor size is

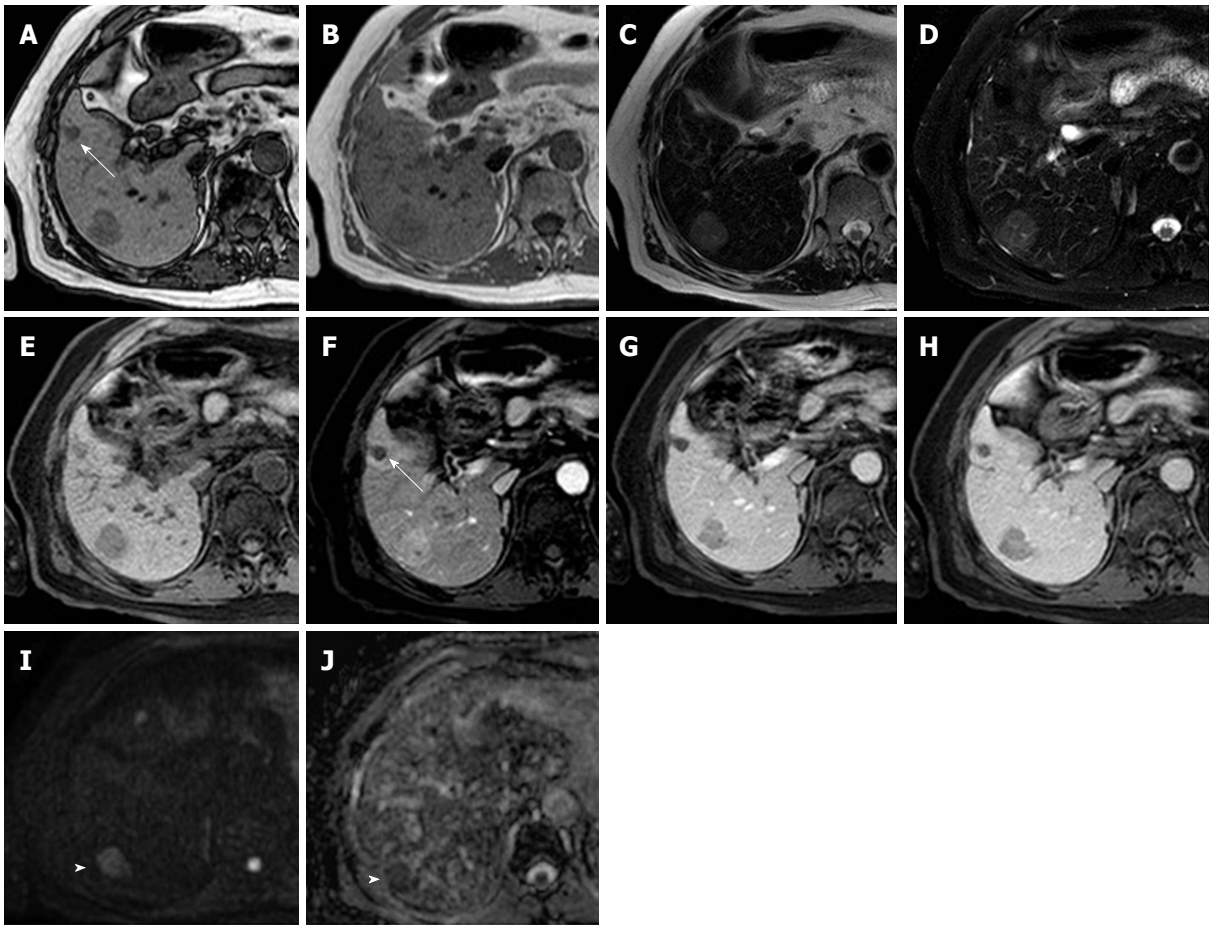

Figure 4 Gd-EOB-DTPA enhanced magnetic resonance images of a 61-year-old patient with hepatocellular carcinoma nodule in the VII segment of the liver. Panels A-B: A single nodule slightly hypointense on the T1-weighted "in phase" sequence (A) with a signal drop in the "out of phase" sequence, as per fat deposition. Panels C-D: On T2-weighted image without and with fat saturation the nodule appears slightly hyperintense. Panels E-H: Dynamic contrast-enhanced images demonstrate the typical contrast behavior of hepatocellular carcinoma: Which appear hypervascular during the arterial phase (F) with wash-out in portal and delayed phase (G-H). Panel I-J: Diffusion weighted image (DWI) shows the hyperintense pattern of the lesion which appear hypointense on the relative apparent diffusion coefficient map (arrowhead). Previously treated lesion with transarterial chemoembolization is recognizable, in panel A-E-F-G-H, at V segment of the liver (arrow). No any restriction of signal intensity is evident on DWI (panel I-J).

the common effect of conventional chemotherapy, loco-regional therapies may lead to stability of tumor size or even an increase in hepatic tumo. Moreover, novel molecular-targeted therapies may alter the morphology of the tumor by affecting its angiogenesis, with unchanged tumor size[77]. Recent studies have shown the possibility to differentiate viable tissue from necrosis on the basis of ADC cut-off values, because necrosis has higher ADC values[78,79]. For patients with HCC treated with Sorafenib, a transient decrease in tumor ADC value approximately 1 month after treatment has been reported to be suggestive of hemorrhagic necrosis; however, a sustained decrease in ADC at 3-mo follow-up may indicate viable tumor or its progression[80]. ADC values in patients with HCC treated with transarterial radioembolization (TARE) have been shown to increase, a finding suggestive of cellular necrosis. Increased ADC values in such cases may be an early marker of treatment response before changes in tumor size are observed[81]. Despite the several attempts to use ADC values in clinical practice, reproducibility of volumetric quantification with diffusion-weighted imaging is not well established. Moreover, there are some technical aspects that need to be considered, like the differences in scanner equipments, the lack of a standardized DWI protocol, the low reproducibility and comparability of ADC measurements among different studies, and finally, the susceptibility of ADC maps to noise and artefacts[64].

MR ELASTOGRAPHY

MR elastography (MRE) is an MRI-based method for the quantitative assessment of liver fibrosis and increased stiffness. This technique is based on the application of mechanical waves (generated through the machine) to the region of interest (the liver). These waves and their wave-length are located in the liver through different elastographic sequences (the most used are gradient-echo sequences with motion-encoding gradients) to obtain different set of images and maps. With two different reconstruction algorithms applied to this set of images, it is possible to obtain a final colored image,

called "confidence map", with different stiffness areas of the liver expressed with different colors that correspond to different in kilo-pascal values (kPa).

Different studies have demonstrated the possibility to use MRE for assessment of mild degree of liver fibrosis[82-84] and to differentiate malignant and benign nodules in the liver[85]. A recent study tried to understand if there was a correlation between HCC stiffness detected with MRE and HCC pathologic features[84]. Tumor stiffness (TS) seemed to be higher in moderate/well differentiated HCC in comparison to poor differentiated HCC (6.5 ± 1.2 kPa *vs* 4.9 ± 1.2 kPa, $P < 0.01$); but at the moment, no correlation is found to liver parenchyma stiffness, vascular invasion, and tumor encapsulation[84].

Another important application of MRE regards the assessment to treatment response and in particular loco-regional treatment [^{90}Yttrium radio-embolization (RE), trans-arterial chemoembolization (TACE), and radiofrequency ablation (RFA)][86].

In two animal studies[87,88], reduction in TS was associated with histologically proven central necrosis[89] and decreased cellular proliferation and moderate induction of apoptosis[88]. In a preliminary study on humans, MRE seems to provide early evidence of therapeutic response, demonstrating that treated tumors have significantly lower TS compared to untreated tumors (3.9 ± 1.8 kPa *vs* 6.9 ± 3.4 kPa, $P = 0.006$) and cirrhotic liver, while intra-tumoral hemorrhage is associated with higher TS. TS seems to relate with visually assessed percentage of necrosis and ER and is more in patients treated with RE[86].

MRE still has the limitation of hepatic iron overload, which can decrease hepatic signal intensity in gradient echo based MRE sequences to unacceptably low levels[83]. On the other hand, MRE enables qualitative and quantitative assessment of TF without the use of gadolinium chelates[86].

Despite some of the limitations of MRE, it remains a promising technique not only for the evaluation of liver fibrosis but also in the spectrum of diagnosis and prognosis of HCC[83,84,86].

RADIOMICS

Radiomics represents the possibility to convert digital medical images (CT, MR, or positron emission tomography images) into high-dimensional data[89]; the hypothesis is that biomedical images contain information that reflects underlying pathophysiology and that these relationships can be revealed *via* quantitative image analyses. MRI based radiomics signature are currently investigated in glioblastoma, breast, and faringeal cancer. Currently, there are no studies about the possibility to use radiomics in the assessment of HCC. Main efforts remain focused on some complex texture analysis, taking in account just few features, which represent a small and impaired part of radiomics data analysis.

Controversial results were obtained from different studies concerning the use of texture analysis in the assessment of HCC[90-93]. The main problems are due to differences in the equipment, contrast phase chosen for the analysis, and type of segmentation (circular ROI *vs* tumor shape ROI, slice analysis vs volumetric ROI analysis). Recently, two studies have been published on the possibility to use complex texture analysis in MRI to assess the malignancy of HCC (Zhou *et al*[94]) or to predict the progression of hypovascular nodules (detected with gadoxetate disodium acid during hepatobiliary phase) into hypervascular HCC lesions[90]. In both studies, volumetric region of interest (VOI) was evaluated.

All these preliminary studies demonstrated that, among the different features assessed with texture analysis, some of them seem to perform better on a specific dynamic phase (arterial or hepatobiliary) and can give useful information. In order to differentiate low grade and high grade HCC[94], "mean intensity value" (a histogram feature) presented significantly larger values in low-grade HCCs than in the high-grade HCCs, and the values of gray-level run-length nonuniformity (GLN) were significantly smaller in low-grade HCCs than in high-grade HCCs.

Moreover, in another study, different histogram metrics showed the possibility to predict the progression of a hypovascular nodule into an HCC[90], using different flip angles and volumetric region of interest.

Radiomics appears to offer a nearly limitless supply of imaging biomarkers that could potentially aid in cancer detection, diagnosis, assessment of prognosis, prediction of response to treatment, and monitoring of disease status[89]. Further studies and validations are required for the performance of the features by themselves, their application according to the different contrast phases available during MRI sequences, and the different MRI equipment.

CONCLUSION

MRI of the liver represents an important tool for the detection and characterization of focal liver lesions and for the evaluation of diffuse liver disease. The main advantages of MRI relies on superior soft tissue contrast, absence of ionizing radiation, and the possibility of performing functional and advanced imaging techniques. Unlike conventional MR imaging sequences, which are usually reported qualitatively based on the varying brightness of tissue, functional MR-imaging techniques offers quantitative data. Among the different functional MR imaging techniques, DWI, MR elastography, and T1-weighted DCE sequences are the most likely to find clinical use at present or in the near future in liver imaging.

MR functional imaging allows for the addition of qualitative and quantitative functional information to conventional anatomic sequences and routine clinical protocols, thereby offering clinicians further comprehensive

information about the biology, behavior, and prognosis about HCC lesions.

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REVIEW

Taking advantage of the potential of mesenchymal stromal cells in liver regeneration: Cells and extracellular vesicles as therapeutic strategies

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Abstract

Cell-based therapies for acute and chronic liver diseases are under continuous progress. Mesenchymal stem/stromal cells (MSCs) are multipotent cells able to migrate selectively to damaged tissue and contribute to its healing and regeneration. The MSC pro-regenerative effect occurs due to their immunomodulatory capacity and their ability to produce factors that promote cell protection and survival. Likewise, it has been observed that part of their paracrine effect is mediated by MSC-derived extracellular vesicles (EVs). EVs contain proteins, lipids and nucleic acids (DNA, mRNA, miRNA, lncRNA) from the cell of origin, allowing for intercellular communication. Recently, different studies have demonstrated that MSC-derived EVs could reproduce, at least in part, the biological effects obtained by MSC-based therapies. Moreover, due to EVs' stability for long periods of time and easy isolation methods they have become a therapeutic option to MSCs treatments. This review summarizes the latest results achieved in clinical trials using MSCs as cell therapy for liver regeneration, the role of EVs in liver physiopathology and the potential of MSC-derived EVs as intercellular mediators and therapeutic tools in liver diseases.

Key words: Mesenchymal stem cells; Extracellular vesicles; Cirrhosis; Liver; Acute damage; Regeneration

Core tip: Cell-based therapies for acute and chronic liver diseases are very attractive strategies. In particular, mesenchymal stem/stromal cells (MSCs) are multipotent cells able to induce protective and pro-regenerative effects in different liver diseases. The mechanism through which MSCs support tissue regeneration is *via* secretion of paracrine factors, and solid evidence supports that part of these effects is mediated by extracellular vesicles (EVs). Therefore, EVs have become an attractive option in the research for new treatments in liver diseases.

Fiore EJ, Domínguez LM, Bayo J, García MG, Mazzolini GD. Taking advantage of the potential of mesenchymal stromal cells in liver regeneration: Cells and extracellular vesicles as therapeutic strategies. *World J Gastroenterol* 2018; 24(23): 2427-2440 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2427.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2427

INTRODUCTION

A diverse set of toxic, metabolic, and inflammatory insults result in liver diseases and imply different degrees of inflammation, apoptosis, and necrosis of parenchymal cells[1-4]. For example, acute liver failure (ALF) is characterized by a massive and sudden death of hepatocytes that lead to abrupt hepatocellular and systemic dysfunction[3]. Similarly, in patients with chronic liver disease an important loss of viable parenchymal cells is observed[1,2,4]. Cirrhosis is caused by diverse chronic liver diseases, such as viral hepatitis and chronic alcoholism[1,2]. Moreover, increases in the prevalence of hypertriglyceridemia, obesity and diabetes in developed countries have resulted in an increase in the incidence of non-alcoholic fatty liver disease (NAFLD)[4,5]. This condition is characterized by a lipid accumulation in the liver that could lead to hepatocytes apoptosis and inflammation. Regardless the liver chronic disease origin, the apoptosis of hepatocytes results in extracellular matrix accumulation that will affect the liver histoarchitecture of liver and ultimately impair its function[4]. It is well known that mesenchymal stem/stromal cells (MSCs) migrate toward injured organs where they can provide tissue protection and promote liver regeneration[6-8]. These properties make MSCs interesting tools to carry therapeutic genes in modern cellular-based therapeutic strategies[6]. It is accepted that the main mechanism through which MSCs support tissue regeneration is *via* secretion of paracrine factors[7,9]. However, solid evidence supports that part of these effects are mediated by extracellular vesicles (EVs)[10]. In this review, we first provide an update on clinical trials using MSCs in different liver diseases; second, the mechanisms involved in the therapeutic effects of MSCs; third, general EVs characteristics and their role in liver diseases, and finally, the role of MSC-derived EVs as therapeutic tools for liver regeneration.

CLINICAL TRIALS INVOLVING THE USE OF MSCS IN LIVER DISEASES

Clinical investigations using MSCs to treat a broad spectrum of degenerative diseases, including liver diseases, are increasing steadily in recent years[11,12]. The first clinical trial using MSCs was started in 2005 and 52 trials are registered up to now (CinicalTrial.gov and reviewed by Tsuchiya 2017[13]). MSCs are obtained from bone marrow in most of the studies, but other sources such as umbilical cord, adipose tissue and menstrual blood has also been tested (Figure 1A). It should be noted that, allogeneic transplantation is more commonly used than autologous (Figure 1B). Between liver diseases, most of the trials are destined to the treatment of liver cirrhosis (Figure 1C) and only 2 of them are in phase II/III (CinicalTrial.gov). Unfortunately, only 22 of 52 registered clinical trials have published their results (Table 1). It is important to mention that MSCs were administered after culture *in vitro* between passages 3 to 6. Regarding the administration route, MSC transplant was performed by peripheral vein[14-28], hepatic artery[29-33], portal vein[15,27] or directly into the spleen[16,34,35]. One study performed on 12 patients showed similar therapeutic effects when MSCs were injected into the spleen or intravenously[17].

In general, MSC administration in patients with different liver pathologies proved to be feasible and safe (Table 1). Regarding their efficacy, studies demonstrated that MSCs exert positive effects on liver function by increase in serum levels of albumin and improving coagulation or decreasing bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (γ-GT)[17-19,21-24,26,29,31,33-35]. In addition, the MELD (Model for End-stage Liver Disease) and Child-Pugh scores were improved after MSC treatment[14-19,23,28-32,34] (Table 1). Furthermore, in some studies it was demonstrated that the application of MSCs not only improved patient's quality of life but also modulated the immune response of patients[22,26,31]. In this sense, a randomized clinical trial using autologous BM-derived MSCs in patients with hepatitis B virus-related liver cirrhosis resulted in an improvement of liver function, an increase in Treg cells, and a decrease in Th17 cells, serum levels of interleukin (IL)-17, tumor necrosis factor alpha (TNF-α) and IL-6; in addition, tumor growth factor beta (TGF-β) levels were increased in comparison with control group[31]. Similar results were reported in a phase I/II clinical trial in patients with primary biliary cirrhosis (PBC) transplanted with allogeneic MSCs that showed a reduction in the number of CD8+ T-cells and an increment of Treg cells and IL-10 serum levels[22].

Recently, Suk *et al*[32] reported a phase II clinical trial comparing the effects of one or two doses of autologous BM-derived MSC therapy with a control group in alco-

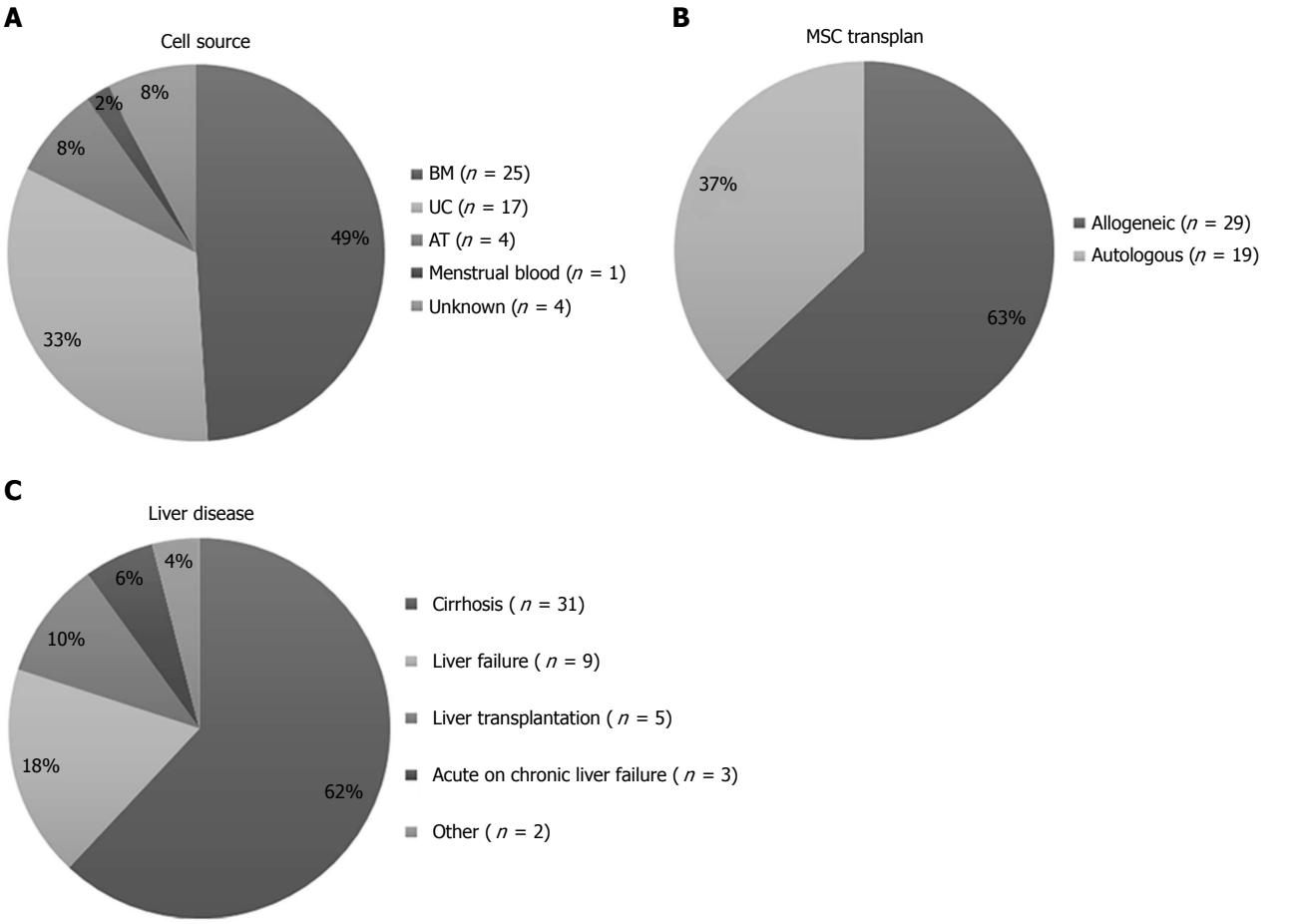

Figure 1 Mesenchymal stem/stromal cells clinical trials in liver disease. A: MSCs clinical trials classified by cell source; B: MSCs clinical trials classified by transplant type; C: MSCs clinical trials classified by liver disease treated. Data from http//www.clinicaltrial.gov. *n*: Number of clinical trials; MSCs: Mesenchymal stem/stromal cells; BM: Bone marrow; UC: Umbilical cord; AT: Adipose tissue.

holic patients. The authors observed that liver fibrosis quantification diminished after cell treatment, although no significant differences in fibrosis area were found between one and two doses. Importantly, no evidence of tumor formation was found during the follow-up in the MSC-treated groups[32].

On the other hand, MSCs were used to diminish hepatocellular damage in acute liver diseases. Recently, a phase II clinical trial in acute-on-chronic liver failure compared the standard medical treatment with one dose of allogeneic BM-derived MSC therapy[28]. In this study, MSC treatment demonstrated to be safe and able to improve bilirubin levels and MELD score. Interestingly, MSC transplant increased survival rate and decreased severe infection events[28].

Long-term immunosuppression is frequently associated with impairment in patients´ quality of life and increased risk of infection or cancer. Therefore, considering their immunomodulatory capacity, MSCs can be used to induce tolerance after liver transplantation. In this regard, Detry *et al*[25] reported that MSC infusion 3 d after liver transplant was feasible, safe and well tolerated. No opportunistic infections or *de novo* cancer were detected in 12 mo follow-up. However, no difference was observed in peripheral blood $CD4^+$ T cell proportion and immunosuppression could not be withdrawn in MSC-treated patients. In addition, the administration of umbilical cord-derived MSCs as therapy to prolong graft-survival in patients with severe ischemic-type biliary lesions after liver transplantation was also evaluated[24]. Six doses of MSCs were infused intravenously every 2-4 wk, and patients were followed-up for 2 years and compared with a prospective cohort of patients treated with standard therapy. Remarkably, MSCs were safe, improved hepatic function, prolonged graft-survival and did not induce cancer or increase microbial infection events[24]. In conclusion, these results demonstrated that MSC therapy is a safe procedure, especially in the field of solid organ transplant where intense and prolonged immunosuppression is required. Moreover, MSC administration could prevent the development of opportunistic infections or *de novo* tumor formation. All considered, the promising information generated in the clinic opens the possibility for further studies to determine the optimal protocol conditions of MSC application needed to induce tolerance after liver transplant.

Application of MSCs for treatment of either acute or chronic liver diseases has a promising future in the clinic. In acute liver diseases, MSCs could have a role decreasing liver damage progression or as a bridge for

Table 1 Mesenchymal stem/stromal cells clinical trials for liver diseases

Ref.	Etiology or disease	Cell source/ origen	Study design (*n*; groups)	Cell administration condition			Phase	Results	Follow up	Side effects
				Dose	*P* value	Route				
Mohamadnejad *et al*[14] 2007	Cirrhosis	BM/Auto	*n* = 4	1, 0.6 × 10^7	2-4	IV	I	MELD ↓	12 mo	None
Kharaziha *et al*[15] 2009	Cirrhosis	BM/Auto	*n* = 8; 4 HBV 1 HCV 1 Alcohol 2 Control	3.5 × 10^7	3-4	PV/IV	I/II	MELD ↓	24 wk	None
El-Ansary *et al*[16] 2010	Cirrhosis	BM/Auto	*n* = 12	10 × 10^6	1	IS/IV	I	MELD ↓; no differences between IS *vs* IV	6 mo	NA
Peng *et al*[29] 2011	Cirrhosis (HBV)	BM/Auto	*n* = 158; 53 MSC 105 Control	3.4-3.8 × 10^8	3	HA	I/II	ALB ↑, MELD ↓	48 mo	None
Amer *et al*[34] 2011	Cirrhosis (HCV)	BM/Auto	*n* = 40; 20 MSC 20 Control	2 × 10^7	NA	IS/IH	I/II	ALB ↑, C.P ↓, MELD ↓	6 mo	Fever (50%), transient shivering (15%)
El-Ansary *et al*[17] 2012	Cirrhosis (HCV)	BM/Auto	*n* = 25; 9 MSC 6 Hep. Diff. 10 Control	1 × 10^6/kg MSC or 40% HLCs and 60% MSCs	5	IV	II	ALB↑, MELD ↓, no differences between HLCs *vs* MSCs.	6 mo	NA
Zhang *et al*[18] 2012	Cirrhosis (HBV)	UC/Allo	*n* = 45; 30 MSC 15 Control	0.5 × 10^6/kg every 4 wk, 3 times	3-4	IV	I/II	ALB ↑, MELD↓, ascites ↓	48 wk	None
Shi *et al*[19] 2012	Acute-on-chronic Liver failure (HBV cirrhosis)	UC/Allo	*n* = 43; 34 MSC 9 Control	0.5 × 10^6/kg every 4 wk, 3 times	3-4	IV	I/II	ALB ↑, PT ↑, MELD ↓, SR↑	72 wk	None
Mohamadnejad *et al*[20] 2013	Cirrhosis	BM/Auto	*n* = 25; 14 MSC 11 Control	2 × 10^8	3-4	IV	II	No beneficial effect	12 mo	None
Wang *et al*[21] 2013	UDCA-resistant PBC	UC/Allo	*n* = 7	0.5 × 10^6/kg every 4 wk, 3 times	4	IV	I/II	ALP↓, γ-GT ↓, quality of life↑ (fatigue↓, pruritus↓)	48 wk	None
Amin *et al*[35] 2013	Cirrhosis	BM/Auto	*n* = 20	10 × 10^6	2	IS	I/II	ALT ↓,AST ↓,BIL ↓, PT ↓, ALB↑, PT↑	24 wk	None
Jang *et al*[30] 2014	Cirrhosis	BM/Auto	*n* = 11	5 × 10^7 every 4 wk, 2 times	4-5	HA	II	C.P ↓, TGF-β ↓, α-SMA ↓, collagen-1 ↓, fibrosis ↓,	20 wk	None
Wang *et al*[18] 2014	UDCA-resistant PBC	BM/Allo	*n* = 10	3-5 × 10^5/kg	3-5	IV	I/II	ALT ↓, AST ↓, γ-GT, BIL ↓, IgM ↓, Tregs ↑, IL-10↑, CD8+T cells↓	12 mo	None
Salama *et al*[23] 2014	Cirrhosis	BM/Auto	*n* = 40; 20 MSC 20 Control	1 × 10^6/kg	0	IV	II	ALT↓, AST↓,BIL↓, ALB↑, PT↑, C.P↓, ascites ↓	26 wk	NA
Xu *et al*[31] 2014	Cirrhosis	BM/Auto	*n* = 56; 27 MSC 29 Control	0.75 × 10^6/kg	NA	HA	II/III	ALB↑, MELD↓, ↑ Tregs/Th17 cell ratio, IL-17↓, TNF-α ↓, IL-6↓, TGF-β ↑	24 wk	NA
Suk *et al*[32] 2016	Cirrhosis	BM/Auto	*n* = 55; 18 MSC (× 1 d) 19 MSC (× 2 d) 18 Control	5 × 10^7 (1 mo post BM asp.) /5 × 10^7 (1 and 2 m post BM asp.)	4-5	HA	II	C.P ↓, fibrosis ↓	12 mo	None
Zhang *et al*[24] 2017	Ischemic-type biliary lesions	UC/Allo	*n* = 82; 12 MSC 70 Control	1 × 10^6/kg; week 1, 2, 4, 8, 12 and 16	4	IV	II/III	BIL↓,ALP↓, γ-GT ↓, graft survival ↑	24 mo	None
Detry *et al*[25] 2017	Liver transplantation	BM/Allo	*n* = 20; 10 MSC 10 Control	1.5-3 × 10^6/kg; day 3 post-transplant	2-3	IV	I/II	No beneficial effect	6 mo	None
Sakai *et al*[33] 2017	Cirrhosis	AT/Auto	*n* = 4	6.6 × 10^5/kg	0	HA	I	ALB ↑, PT↓	1 mo	None
Shi *et al*[26] 2017	Liver transplantation	UC/Allo	*n* = 20; 14 MSC 13 Control	1 × 10^6/kg; every 4 wk, 3 times	3-4	IV	I	ALT↓, AST↓, BIL ↓, improve liver allograft histology, acute rejection↓ (↑ peripheral Tregs, ↑ Tregs/Th17 cell ratio).	12 wk	None
Hartleif *et al*[27] 2017	Pediatric liver transplantation	BM/Allo	*n* = 7	1 × 10^6/kg; day 0 and day 2 post-transplantation	2-3	PV/IV	I	NA	24 mo	None

Lin *et al*[28] 2017	Acute-on-chronic Cirrhosis (HBV)	BM/Allo	n = 110; 56 MSC 54 Control	1-10 × 10^5 cells/kg; 1/wk, 4 wk	5-6	IV	I/II	MELD↓, SR↑, infections↓	24 wk	None

P: Passage; BM: Bone Marrow; Allo: Allogeneic; Auto: Autologous; IV: Intravenous Infusion; MELD: Model for end-stage Liver Disease; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; IS: Intrasplenic; IH: Intrahepatic; NA: Not available; HA: Hepatic artery; CP: Child-Pugh score; HLC: Hepatocyte-like cells; UC: Umbilical cord; SR: Survival rate; Cr: Creatinine; BIL: Bilirubin; PBC: Primary biliary cirrhosis; UDCA: Ursodeoxycholic acid; Hep. Diff.: Hepatocyte differentiated; PT: Prothrombin time; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; γ-GT: γ-glutamyltrans peptidase; Asp: Aspiration.

liver transplant. In chronic liver diseases, MSCs could contribute to decrease liver damage, to ameliorate the degree of fibrosis, and even to avoid the need for transplant in some particular cases. On the other hand, in the post-transplant setting, MSC therapy could extend the graft survival and/or decrease the amount of immunosuppression. However, it is extremely important to understand that therapeutic potential of MSCs is an open question, and the information regarding source of MSCs, culture conditions, pre-condition protocols before cell transplantation, administration route, number of doses, and time of treatment are very heterogeneous and standardization is needed.

MECHANISM OF ACTION OF MSCS IN LIVER REGENERATION

As detailed above, is necessary to understand the mechanisms that mediate MSCs therapeutic effects prior to continuing with its clinical application. The understanding of MSC biology has grown considerably. In the last decade, many mechanisms involved in their regenerative potential have been identified. These mechanisms involve, at least in part, migration toward injured tissues, immunomodulatory properties, differentiation and/or secretion of regenerative factors, which induce cell survival and proliferation[7,9,36,37]. It has been described that MSCs can be recruited to inflamed tissue by classic mechanism of blood stream migration: Rolling, adherence to endothelium and transmigration[8,36]. The injured liver produces signals that induce migration and homing of different cell types[38,39]. Both, chronic and acute liver injury induce apoptosis and necrosis of hepatocytes and/or cholangiocytes, infiltration of leucocytes, monocytes and activation of Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HeSCs)[2,3,40]. In this context, MSCs could be attracted by several chemokines, cytokines and factors secreted by the damaged liver microenvironment, such as IL-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), growth-regulated protein (GRO), TNF-α, TGF-β1, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor 1 (SDF-1) among others. Receptors for these chemoattractants were found to be expressed in MSCs allowing them to migrate to the injured liver[11,36].

The differentiation potential of MSCs has been considered one of the advantages for their regenerative application[7,41-43]. By definition, MSCs give rise to cells of mesodermal lineages including osteoblasts, adipoblasts and chondroblasts[44]. Furthermore, there are many reports demonstrating that MSCs might differentiate to ectodermal (neurons), endodermal (hepatocytes) as well as other mesodermal lineages (cardiomyocytes), but this field remains very controversial[7,41]. In this context, a putative mechanism postulated for liver regeneration was the MSC differentiation into hepatocyte-like cells[45]. A number of differentiation *in vitro* protocols were explored in MSCs obtained from bone marrow, adipose tissue or umbilical cord demonstrating that MSC-derived cells acquired characteristic markers and functions of immature and mature hepatocytes (reviewed in Fiore *et al*[7]). In fact, a clinical trial compared the effect of autologous BM-MSCs undifferentiated or differentiated into hepatocyte-like cells in patients with cirrhosis. Despite the improvement of liver function, there was no significant difference between undifferentiated or differentiated MSC-based treatment[17]. However, difficulties to study and reproduce the *in vitro* differentiation and *in vivo* tracking after their administration, makes the MSC differentiation to hepatocytes a controversial field. This difficulty is due, at least in part, to unspecificity and/or unreliability of methods/trackers/reagents frequently used for the identification of cells or the phenotype they might acquire after their transplantation[46,47]. It should be noted that track able MSCs by GFP expression is detectable in the liver up to 7 d after transplant and then the signal rapidly decreases in animal models[48]. Moreover, some evidence indicated that in spite of the MSC homing to the liver, the rate of differentiation to hepatocyte-like cells is very low (less to 1%)[49,50]. Together, this information demonstrates that the main regenerative effect of MSCs cannot be explained by the differentiation to hepatocyte-like cells.

Broad scientific consensus states that the regenerative effect of MSCs is due to paracrine mechanisms. The versatility of MSCs makes them able to differentially express factors depending on the surrounding microenvironment[51]. In the context of liver fibrosis, it was observed that MSCs produce high levels of anti-apoptotic growth factors such as SDF-1, VEGF, hepatocyte growth factor (HGF) and insulin-like growth factor (IGF)-I[9]. Also, the release of HGF, fibroblast growth factor (FGF), IL-6, fibrinogen and TGF-α can induce hepatocyte proliferation[51]. In addition, HGF

and epidermal growth factor (EGF) can induce hepatic progenitor cell proliferation and differentiation[52] and VEGF increases angiogenesis, an important event for liver regeneration[53]. Moreover, IL-10, HGF and IGF-I produced by MSCs can reduce fibrogenesis by inhibition of HeSCs activation and proliferation[7,9]. MSCs also secrete factors involved in extracellular matrix remodeling and chemokines that attract immune cells which could modulate their function[51]. In this way, *in vivo* administration of conditioned medium (CM) obtained from MSC cultures can be effective to reduce liver injury. It was reported that the administration of MSC-derived CM significantly improved short-term survival in a D-galactosamine-induced rat model of fulminant hepatic failure[54,55]. Furthermore, MSC-CM therapy had great inhibitory effects on hepatocellular death reducing hepatocyte apoptosis in a 90%. In addition, an increase in liver regeneration programs and number of proliferating hepatocytes was observed[55]. Subsequently, it was demonstrated that EVs, present in CM, are partly responsible of the therapeutic effects of MSCs[10,56].

EXTRACELLULAR VESICLES

Although, EVs were described as intracellular mediators many years ago, they have recently generated great interest as therapeutic and diagnostic tools. Initially, "extracellular vesicle" was used to refer to all kind of vesicles released by cells. Nevertheless, the increased knowledge of their biology allowed to distinguish between different types of vesicles[57]. Exosomes (50-100 nm in diameter) are homogenous and the largest family of EVs and are different from microvesicles (100-1000 nm) and apoptotic bodies (500-2000 nm) in size and biogenesis[58]. While exosomes are originated from multivesicular bodies (MVBs), microvesicles are originated directly from plasma membrane and released to extracellular space[58]. At present, vesicles can only be fractioned according to their sizes and no specific markers have been described. Due to a large heterogeneity in methods of isolation and terminology in the past published results, in this review we will refer to exosomes and microvesicles as "EVs". In order to define a minimal criteria for EV characterization, the International Society for Extracellular Vesicles (ISEV) suggests a semi-quantitative analysis for typical protein marker, such as CD9, CD63, CD81, Alix or TSG101, size analysis and morphology examination[59]. In addition to these specific proteins, EVs contain a large number of proteins (growth factors, cytokines, vesicles proteins), DNAs, mRNAs, microRNAs (miRNAs), long non-coding RNAs (lncRNAs)[57,59]. An interesting characteristic of EVs is that they can be charged with specific components of the cell of origin, and this "cargo" could be modified by different stimuli and microenvironment conditions[58]. As mentioned above, it has been demonstrated that EVs are involved in the paracrine effects of MSCs, but as EVs are implicated in many intercellular communications, we will first describe their implication in liver diseases.

EVS IN LIVER DISEASES

EVs have been implicated in a number of physiological and pathophysiological processes, such as immune response, angiogenesis, tissue regeneration, tumorigenesis/metastasis and neurodegenerative diseases[60-62]. In patients with primary biliary cirrhosis it was demonstrated that serum exosomes are taken up by peripheral monocytes and dendritic cells, resulting in the up regulation of co-stimulatory molecules[63]. Interestingly, serum circulating EVs present different miRNA composition in cirrhotic patients when compared with healthy controls[63,64]. Recent findings demonstrated that EVs are implicated in viral hepatitis, drug-induced injury, alcohol injury, non-alcoholic steatohepatitis and biliary injury[65,66].

EVs derived from parenchymal cells

Exosomes transport a variety of macromolecules that could act as signals between donor and recipient cells. *In vitro* studies demonstrated that liver parenchymal cells produce EVs that are involved in many physiological and pathophysiological processes[65,66]. For instance, it was demonstrated that there is an increase in the number of circulating exosomes with proliferative effect on hepatocytes after ischemia/reperfusion injury[67]. Nojima *et al*[67] reported that EVs derived from hepatocytes, but no other liver cells can induce hepatocyte proliferation *in vitro*. It should be noted that hepatocyte-derived EVs exert their effect in a dose-dependent manner. Furthermore, their administration in mice under ischemia/reperfusion liver injury or after 70% hepatectomy promotes hepatocyte proliferation and liver regeneration. Similarly, Herrera *et al*[68] showed that hepatic progenitor cell-derived EVs promote hepatocyte proliferation, suppress cell death, and accelerate liver regeneration in rats after hepatectomy. The author suggested that this effect is mediated by the delivery of RNA by EVs to target cells. It is worth mention, that current data demonstrated that hepatocyte-derived exosome properties are mediated by fusion with target hepatocytes transferring their cargos[67,69,70]. For example, hepatocyte-derived EVs transfer neutral ceramidase and sphingosine kinase 2 (SK2) within hepatocytes resulting in the induction of sphingosine-1-phosphate (S1P), a demonstrated promoter of cell proliferation[67] (Figure 2). Further studies of the same group showed that CXCR1 is required for packaging of SK2 into exosomes and CXCR2 regulates neutral sphingomyelinase activity and there by neutral ceramidase production[71].

On the other hand, hepatocyte-derived EVs in a nonalcoholic steatohepatitis (NASH) model interact with macrophages inducing an inflammatory phenotype[72] (Figure 2). Activation of the death receptor 5 (DR-5) expressed on hepatocytes by free fatty acid induces

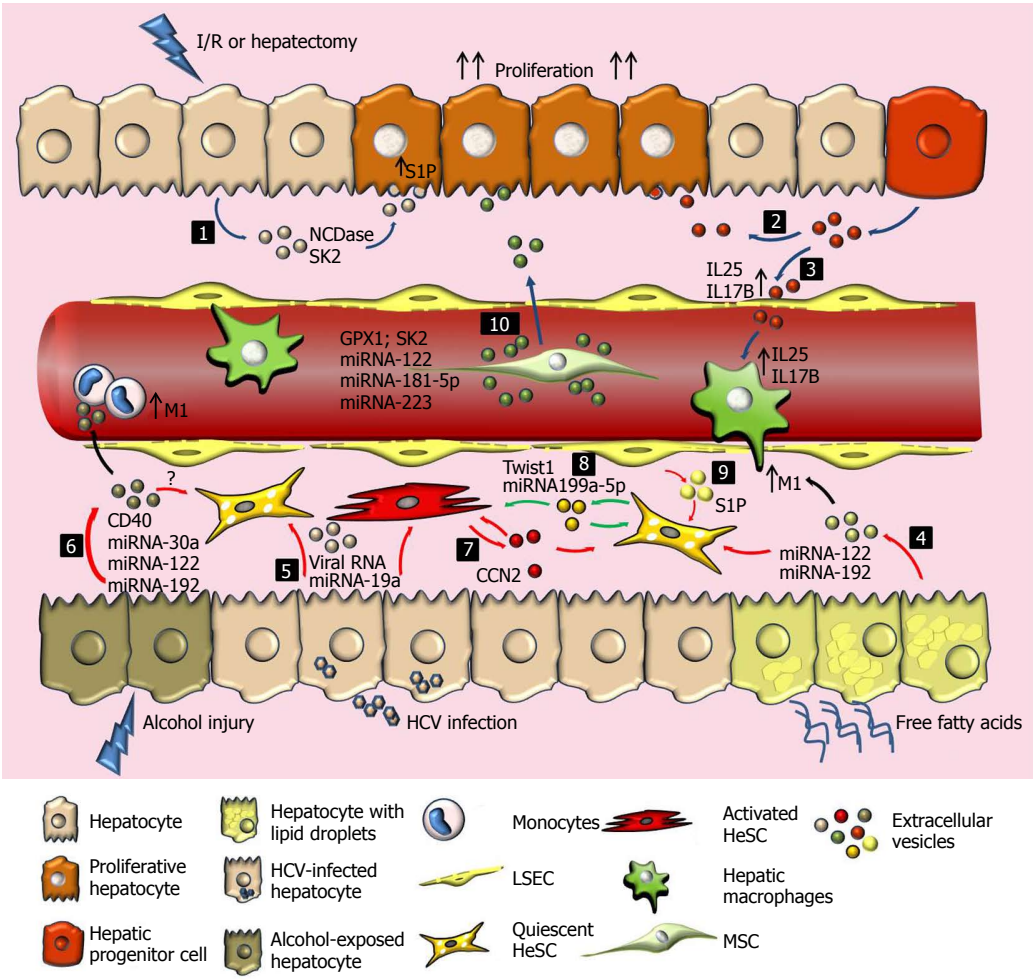

Figure 2 Extracellular vesicles as paracrine mediator in liver disease and therapeutics potential of mesenchymal stem/stromal cells. After ischemia reperfusion injury (I/R) or hepatectomy, hepatocytes (1) HPCs (2) release EVs with the ability to induce hepatocyte proliferation. (3) HPC-derived EVs stimulate LSECs and macrophages production of proliferative cytokines such as IL25 and IL17B. (4) On the other hand, free fatty acids induce the production of hepatocyte-derived EVs that result in the activation of quiescent HeSCs and pro-inflammatory macrophages (M1). (5) During chronic hepatitis C virus infection, EVs secreted by HCV-infected hepatocytes induce activation of HeSCs. (6) EVs secreted by hepatocytes after alcohol injury (containing CD40L and miRNAs) induce activation of monocytes and HeSCs. It seems to be a balance between EVs derived from active or quiescent HeSCs that promotes or inhibits fibrogenesis. Activated HeSC-derived EVs induce activation of quiescent HeSCs trough CCN2 (7) and quiescent HeSCs inhibit activated HeSCs transferring Twist1 or miRNA199a-5p (8). LSEC-derived EVs could also regulate HeSC activation (9). MSC-EVs induce hepatocyte proliferation, reduce oxidative stress and apoptosis, and modulate inflammatory response by carrying GPX1 or SK2 (10). Engineered MSC-EVs transferring miRNA-122, miRNA 181 5p and miRNA-223 have potential effects. The effects of MSC-EVs on HeSCs, hepatic macrophages, LSEC and infiltrated cells populations remain poorly explored. Green arrows: Inactivation of HeSCs; Red arrows: Activation of HeSCs; Blue arrow: Proliferative effect; Colors spots represent EVs from different cell origin; NCDase: Neutral ceramidase; SK2: Sphingosine kinase 2; S1P: Sphingosine-1-phosphate; IL: Interleukin; SK1: Sphingosine kinase 1; CCN2: Connective tissue growth factor; Twist1: Basic helix-loop-helix transcription factor; GPX1: Glutathione peroxidase 1; HCV: Hepatitis C virus; EVs: Extracellular vesicles.

there lease of EVs that could stimulate IL-1β and IL-6 expression on macrophages[72]. On the same line, EVs derived from palmitic acid (PA) stimulated hepatoma cells (Huh7 and HepG2) induce a profibrogenic phenotype on HeSCs[73]. Moreover, the activation of HeSCs seems to be related with the presence of miRNA-122 and miRNA-192 in the EVs[73] (Figure 2). Similarly, an increase in the number of circulating exosomes in mice is observed after chronic alcohol consumption[74]. Furthermore, *in vitro* incubation of hepatocytes with ethanol not only increases the release of exosomes but also allows monocyte activation through miRNA-122 horizontal transfer[74,75]. Remarkably, circulating EVs obtained from patients with alcoholic hepatitis show higher levels of miRNA-30a, miRNA-192 and, in particular, miRNA-122 than those obtained from heathy donors[74] (Figure 2).

EVs have also been implicated in horizontal transfer of information in chronic hepatitis C infection between hepatocytes and HeSCs. Devhare *et al*[76] demonstrated that EVs derived from HCV-infected hepatocytes transfer viral RNAs that increases the expression of profibrogenic markers on HeSCs. Interestingly, these EVs carry miR-19a that activates the STAT3 signaling, enhancing the expression of TGF-β and IL-6. Importantly, miR-19a up-regulation was observed in HCV-infected hepatocytes and in sera of chronic HCV patients with fibrosis[76,77] (Figure 2). Moreover, Seo *et al*[78] demonstrated that EVs are implicated in HeSCs activation *via* toll-like receptor 3 with the subsequent activation of γδ T cell population exacerbating liver fibrosis. Regarding other

liver parenchymal cells, the role of cholangiocyte-derived EVs has been less studied. However, the presence of circulating EVs with altered miRNA composition and immunostimulatory functions on patients with PBC could suggest a role of cholangiocyte-derived EVs on this disease[63,64].

EVs derived from non-parenchymal cells

Non-parenchymal cells, including LSECs, Kupffer cells, lymphocytes, and HeSCs play a critical role in many liver diseases and use EVs for communication with neighbor cells during liver damage[65,66]. For instance, connective tissue growth factor (CCN2), a pro-fibrogenic mediator, is packaged into EVs produced by activated HeSCs. Then, exosome CCN2 can be delivered to other quiescent or activated HeSCs to induce trans-activation[79]. On the other hand, EVs derived from quiescent but not from activated HeSCs transport Twist 1 that suppress CCN2 on target cells through miR-214 induction[80]. Furthermore, Chen *et al*[81] showed that miR-199a-5p is loaded in quiescent HeSCs-derived EVs and also regulates CCN2 expression and activity on target cells (Figure 2). It seems to be a balance between EVs derived from active or quiescent HeSCs that promotes or inhibits liver damage. In addition, LSECs are known to maintain the HeSC quiescence through direct cell-to-cell contact and paracrine factors secretion[82]. Wang *et al*[83] demonstrated that LSEC-derived EVs have the ability to transfer Sphingosine kinase 1 (SK1) to regulate HeSC activation. In contrast, Ichinohe *et al*[84] demonstrated that progenitor hepatic cell-derived EVs stimulate LSECs and Kupffer cells to produce IL17B and IL25 resulting in proliferation of small progenitor hepatic cells (SPHCs) and liver regeneration (Figure 2). These findings evidence the importance of EVs derived from LSECs in affecting the activation state of neighboring cells. However, the mechanisms by which exosomes reach and attach target cells are not well understood. It has been reported that endocytosis of LSEC-derived EVs is mediated by the interaction between exosomal fibronectin and its ligand on the surface of HeSCs[83]. Additionally, integrin $\alpha v\beta 3$ or $\alpha 5\beta 1$ and heparan sulfate proteoglycan are ligand for EVs-HeSCs interaction and allow information transfer from endothelial cells[85].

Other important non-parenchymal cells involved in fibrogenesis are Kupffer cells and infiltrating macrophages. These cells are key players not only in fibrogenesis but also in fibrosis resolution and regeneration[86,87]. In alcoholic liver injury, hepatocyte-derived EVs enriched in miR-122 sensitize macrophages to LPS and induce their production of pro-inflammatory cytokines[75]. Moreover, CD40L presence on hepatocyte-derived EVs during alcoholic hepatitis promotes macrophage activation and the switch to a pro-inflammatory profile[88,89]. In contrast, *in vitro* exposure of monocytes to alcohol increased their release of EVs, which in turn induce an anti-inflammatory M2 profile of naïve monocytes[90]. Interestingly, EVs derived from alcohol exposed monocytes contain high levels of miR-27a that is known to induce M2 polarization[90,91] (Figure 2).

In summary, it has been determined that EVs play a key role on the pathophysiological response to liver damage. EVs allow the interaction between parenchymal cells and non-parenchymal cells, mainly HeSCs, Kupffer cells and LSECs, mediating their anti/pro-fibrogenic state. The role of EVs in cell-to-cell communication during liver damage and the transfer of molecules, proteins and miRNAs is gaining importance in the field. Furthermore, this mechanism can be exploited for new therapeutic approaches or used as biomarkers in non-invasive methods. In line with this, it has recently been reported that MSCs release high levels of EVs that can mediate part of their therapeutic effects. Thus, considering the key role of EVs in liver cell communication, MSC-derived EVs (MSC-EVs) could be studied as a new therapeutic approach for hepatic regeneration strategies.

MSC-EVS AND THEIR POTENTIAL FOR LIVER REGENERATION

As described above, the main mechanism by which MSCs support the repair and regeneration of injured tissues is by releasing paracrine factors[7]. Recently, this paracrine mechanism was described to be partially mediated by EVs released by MSCs[10,56]. *In vitro* assays demonstrated that MSC-EVs induce hepatocyte proliferation and dedifferentiation into progenitor oval cells[92,93]. Therapeutic effects of MSC-EVs were demonstrated in pre-clinical models of acute kidney injury[94], and then in pathologies of heart, brain, kidney, muscle and liver[95].

Li *et al*[96] demonstrated that MSC-EVs reduced the degree of hepatic injury, collagen deposition and inflammation in mice with fibrosis induced by carbon tetrachloride (CCl_4). The antifibrotic effect observed by MSC-EVs is mediated by the inactivation of TGF-β1/Smad signaling pathway. Moreover, a reversion of the epithelial-to-mesenchymal transition (EMT) both *in vivo* and *in vitro* was observed after the EV treatment[96] (Figure 2). Therefore, EVs derived from human MSCs were effective in mice demonstrating that they preserve at least part of the immunomodulatory properties of the cells of origin.

As for liver fibrosis, the therapeutic capacity of MSC-EVs was also assessed in acute models of liver injury. Cheng *et al*[97] studied the effect of EVs derived from menstrual blood MSCs in a galactosamine/LPS mice model. MSC-EVs were able to enhance animal survival and reversion of liver failure through hepatocyte apoptosis inhibition and systemic inflammation reduction. In addition, *in vitro* assays showed that EVs are taken up by AML12 cells (hepatocyte cell line) resulting in the inhibition of apoptosis induced by galactosamine/LPS[97]. Similar results were observed by Haga *et al*[98] using EVs

Table 2 Mesenchymal stem/stromal cells-derived extracellular vesicles in experimental models of liver disease

Ref.	EVs Isolation/characteristics	Experimental model	Protocol	Biological effects
Haga *et al*[98] 2016	Ultracentrifugation Size: 46-116 nm $Alix^+$ $CD9^+$, $CD81^+$	C57Bl mice. ALF, i.p. 20 mg/body D-GalNAc + 0,3 mg/body TNF-α	2×10^8 to 2×10^{10} i.p./i.v.	↑ Survival, ↑ F4/80, ↑ inhibitor MMP-1 and IL-6, ↓ inflammation and apoptosis, ↓ ALT/AST, ↓ ALP, ↓ EGF, SCF, IFN-γ, IP-10, IL-1α, MIP-3, MCP-1/3
Yan *et al*[100] 2016	Ultracentrifugation Size: 30-100 nm $CD9^+$, $CD61^+$, $CD63^+$	BALB/c-$^{nu/nu}$ mice, i.p. CCL_4-induced ALF, 0.15-0.35 mL/kg	8, 16, and 32 mg/kg i.v./oral	↓ Oxidative stress and apoptosis Induces ERK1/2 phosphorylation and Bcl2 expression Inhibits IKKB/NFκB/ casp9/3 pathway
Tan *et al*[99] 2014	TFF, 100.kDa MWCO filter Size: 55-100 nm	C57BL/6 mice. CCL_4-induced ALF, i.p. 0.05 mL CCL_4/kg	0.4 μg (100 μL) i.s.	↑ Cell viability: TAMH, THLE-2, and Huh-7 ↑ Hepatocytes proliferation ↓ ALT/AST ↓ Casp 3/7 ↑ antiapoptoticBcl-xL
Chen *et al*[97] 2017	Centrifugation and Exoquick-TC Size: 30-100 nm $CD63^+$ and $tsg101^+$	C57BL/6 mice. ALF, i.p. D-GalNAc 800 mg/kg and LPS 50 μg/kg	1 μg/μL i.v.	↑ Liver function and survival ↓ Apoptosis ↓ TNF-α, IL-6 and IL-1 ↓ Casp-3 ↓ Necrosis and inflammation
Nong *et al*[101] 2016	Ultracentrifugation and ultrafiltration Size: 50-60 nm $CD9^+$, $CD63^+$ and $CD81^+$	Rats I/R injury	600 μg suspended in 400 μL of PBS i.v.	↑ GSH, GSH-PX and SOD ↓ AST/ALT ↓ TNF-α, IL-6 and HMGB1 ↓ Casp-3 and Bax
Du *et al*[93] 2017	Centrifugation and filtered by 0, 45-μm PVDF filter ExoQuick Size: 100-200 nm $Alix^+$, $CD63^+$ and $CD81^+$	C57 mice I/R injury	2.5×10^{12} particles in 500 μL of PBS i.v.	↑ Hepatocytes proliferation ↑ SK activity and S1P formation. Hepatoprotective and proliferative effect abolished by the inhibition of SK or S1P receptor 1

i.p.: Intraperitoneal injection; i.v.: Intravenous injection; i.s.: Intrasplenic; MMP: Metalloproteinases; CCL_4: Corbon tetrachloride; D-GalNAc: N Acetylgalactosamine; IL: Interleukins; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; EGF: Epidermal growth factor; SCF: Stem cell factor; TNF: Tumor necrosis factor; IFN: Interferon; IP: Inducible protein; MIP: Macrophage inflammatory protein; MCP: Monocyte chemotactic protein; ERK: Extracellular signal-regulated kinase; Bcl: B-cell lymphoma; TFF: Tangential flow filtration; MWCO: Molecular weight cut-off; ALF: Acute liver failure; TAMH: Transgenic mouse hepatocyte; THLE: T-antigen immortalized human liver epithelial; Casp: Caspase; GSH: Glutathione; GSH-PX: Glutathione peroxidase; SOD: Superoxide dismutase; HMGB: High mobility group box; PBS: Phosphate-buffered saline; SK: Sphingosine kinase; S1P: Sphingosine 1-phosphate.

derived from bone marrow MSCs in an animal model of acute liver failure. A relevant finding was the preservation of the biological activity of cryopreserved-EVs m up to 3 mo, indicating the stability of EVs[98].

It is known that induction of oxidative stress in the liver results in severe hepatic diseases by inducing cell apoptosis. A protective and proliferative effect of MSC-EVs in a lethal mice model induced by a single dose of CCl_4 has been reported[99]. In addition, MSC-EVs inhibited hepatocyte apoptosis induced by acetaminophen and H_2O_2 and increased cell viability *in vitro*[99]. In this line, Yan *et al*[100] demonstrated that EVs derived from umbilical cord MSCs induced an antioxidant effect on hepatocytes. Glutathione peroxidase 1 (GPX1) delivered on MSC-EVs reduces the reactive oxygen species (ROS) intracellular levels and inhibits the oxidative stress-induced apoptosis *in vitro* and *in vivo.* Remarkably, authors demonstrated that a low dose of EVs (16 mg/kg of body weight) resulted in similar effects either by tail vein administration or oral gavage. In addition, Nong *et al*[101] tested the effect of EVs generated from MSC-derived Induced pluripotent stem cells (iPSCs) (MSC-iPSC-EVs) in hepatic ischemia-reperfusion (I-R) injury models. *In vivo* administration of MSC-iPSC-EVs in I-R injury mice model resulted in a decrease of oxidative stress response and apoptosis, and an increase of hepatocyte proliferation (Figure 2).Consistently, an amelioration of hepatic damage and inflammatory response was observed after EV treatment. It should be noted that MSC-iPSC-EVs keep the characteristics of EVs usually obtained from tissue-derived MSCs (bone marrow, adipose tissue and umbilical cord)[101]. In addition, it has been reported that MSC-iPSC-EVs could directly fuse with hepatocytes increasing the activity of sphingosine kinase (SK1). Moreover, the increase in SK activity will in turn increase the sphingosine-1-phosphate (S1P) levels affecting hepatocyte proliferation[93] (Table 2).

Although there is a great therapeutic potential of MSC-EVs in liver protection and regeneration, it is mandatory to understand the mechanisms involved in their biological effects. One key point of research is to know the bio distribution of EVs after systemic administration *in vivo*. *In vitro* assays showed that EVs are taken up *via* integrin mediated endocytosis by target liver cells[67,85,90,97]. In addition, MSC-EV biodistribution after intravenous (IV) administration shows EVs uptake as fast as 3 to 6 h after injection in liver, spleen and lung cells[97]. Furthermore, Haga *et al*[98] reported that liver, spleen and lung from mice with fulminant hepatitis exert a higher uptake of MSC-EVs in comparison with organs from healthy mice. However, since these experiments have been carried out using lipophilic tracers, confirmation of these results with

specific markers is necessary. The MSC-EV survival in circulation after being administered and the recognition pathways used by the target cells need to be studied in depth. Moreover, this knowledge could help to define the better scheme of doses, the time of treatment and the route of administration depending of the type of liver damage. In addition, conformation details of proteins and nucleic acids loaded in the EVs is required.

ENGINEERING MSC-EVS FOR LIVER REGENERATION

Recently, an increased interest has been shown by the research community to improve the efficiency and potency of EVs loading specific cargos[102]. As described above, the EVs produced by MSCs have useful properties that would allow them to be used as therapies in different liver pathologies[62], and advances in nanomedicine would allow to improve the technology to generate more effective EVs[102,103]. Thereby, engineered EVs can be generated with strategies based on covalent surface chemistry, hydrophobic insertions or membrane permeabilization. Moreover, modification of the parental cells through metabolic labeling, genetic modification or by insertion of exogenous material could also produce modified EVs. These engineered EVs could carry specific DNAs, mRNAs or non-coding RNAs to the specific cell[102].

Lou *et al*[104] demonstrated that miRNA-122 expression in MSCs by lentiviral infection increased their therapeutic effect in a fibrosis model in mice. MiR-122 positively regulates proliferation and trans differentiation of HeSCs having an important role in liver fibrogenesis[105]. Authors demonstrated that *in vitro* miRNA-122 is transferred to HeSCs through MSC-EVs resulting in the regulation of genes involved in collagen maturation and cell proliferation[104]. Another strategy uses EVs derived from MSCs with transient expression of miRNA-181-5p as a therapeutic option in a liver fibrosis model[106]. As for miRNA-122, miRNA-181-5p was delivered by MSC-EVs reducing the fibrosis and the inflammatory state of fibrotic mice[106]. Moreover, *in vitro* experiments show that after reaching the target cell, miRNA-181-5p binds to3′-UTR of STAT3 and Bcl-2, which in turn down regulates TGF-β1 expression and induces autophagy in HeSCs[106]. Finally, in a model of autoimmune hepatitis, a cytoprotective effect of MSC-EVs engineered to charge miRNA-223 was observed. The *in vitro* experiments demonstrated that miRNA-223 levels were increased in hepatocytes after their incubation with the engineered EVs[107]. Similar results were observed *in vivo* with an increase of this miRNA in the liver and a reduction of its target gene NLRP3, and therefore a decrease in hepatocyte apoptosis[107] (Figure 2).

In summary, more information is needed not only to develop more efficient therapies for liver diseases based on MSC-EVs but also to engineer them to increase their efficacy and potency.

CONCLUSION

MSCs-based therapy has emerged as a potent and innovative treatment for acute and chronic liver diseases. The safety and feasibility observed in the early clinical trials using MSCs have increased the interest to translate the use of these cells to the clinic. Moreover, pro-regenerative results and an improvement in the life quality of patients were observed. In ALF, MSCs could have a role decreasing liver damage progression due their immunomodulatory properties. In chronic liver diseases, MSCs could contribute to decrease liver damage and to ameliorate the degree of fibrosis. Even more, in both case MSC treatment could not only delay the transplant but also to avoid it in some particular cases. In addition, in the post-transplant setting, MSC therapy could extend the graft survival and/or decrease the amount of immunosuppression required. Although the main mechanism by which MSCs support the repair and regeneration of injured livers is by releasing paracrine factors, strong evidences demonstrated that this paracrine mechanism is mediated by EVs released by MSCs. Therefore, due to EVs' stability for long periods of time and easy isolation methods they have become a therapeutic option to MSCs treatments in liver diseases. At present, EVs are strongly explored for therapeutic or diagnostic application, and more information is needed to develop more efficient tools for liver diseases based on MSC-EVs. However, it is important to understand that therapeutic potential of MSCs or its EVs is still a matter of debate. In addition, standardization of source of MSCs, culture conditions, pre-condition protocols for cell transplantation, administration route, doses and time of treatment is required. Nevertheless, considering that development of new therapeutic approaches for liver diseases is urgent, MSCs emerge as potent innovation. Thus, take advantage of the therapeutic potential of MSCs as promising tool for liver regeneration could attend to an important worldwide human health problem.

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REVIEW

Feasibility of using marginal liver grafts in living donor liver transplantation

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Abstract

Liver transplantation (LT) is one of the most effective treatments for end-stage liver disease caused by related risk factors when liver resection is contraindicated. Additionally, despite the decrease in the prevalence of hepatitis B virus (HBV) over the past two decades, the absolute number of HBsAg-positive people has increased, leading to an increase in HBV-related liver cirrhosis and hepatocellular carcinoma. Consequently, a large demand exists for LT. While the wait time for patients on the donor list is, to some degree, shorter due to the development of living donor liver transplantation (LDLT), there is still a shortage of liver grafts. Furthermore, recipients often suffer from emergent conditions, such as liver dysfunction or even hepatic encephalopathy, which can lead to a limited choice in grafts. To expand the pool of available liver grafts, one option is the use of organs that were previously considered "unusable" by many, which are often labeled "marginal" organs. Many previous studies have reported on the possibilities of using marginal grafts in orthotopic LT; however, there is still a lack of discussion on this topic, especially regarding the feasibility of using marginal grafts in LDLT. Therefore, the present review aimed to summarize the feasibility of using marginal liver grafts for LDLT and discuss the possibility of expanding the application of these grafts.

Key words: Marginal liver grafts; Living donor liver transplantation; Liver transplant waiting lists; Small-for-size grafts; Older donors; ABO-incompatible; Steatosis; Chronic hepatitis

Core tip: There are few reviews concerning the feasibility of using marginal liver grafts in living donor liver transplantation (LDLT). We reviewed more than 300 articles, summarized new findings, and confirmed that marginal grafts are a feasible option for expanding options for patients on liver transplant waiting lists in emergency situations in LDLT (*e.g.*, liver failure or hepatic encephalopathy). However, such grafts place the recipients at greater risk for adverse events. Although some indispensable treatments are needed to address the deficiencies of these grafts, recipients can receive a favorable prognosis, similar to that of patients who receive standard liver grafts, under these treatments.

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INTRODUCTION

The high mortality of patients on waiting lists due to the shortage of cadaveric donors is a major challenge in liver transplantation (LT)[1]. This challenge has led to the emergence of living donor liver transplantation (LDLT) after the first successful procedure in 1989[2,3]. However, following a sharp increase in recipients who suffer from emergency situations, the wide gap between the demands of patients and suitable living donors is gradually increasing[4,5]. Therefore, the transplantation community has focused on the search for strategies to increase the pool of available liver grafts, including the use of organs that were previously considered "unusable" by many and often labeled "marginal" organs[6].

An accepted definition of marginal donors remains unclear in LDLT. These expanded-criteria grafts have the potential to increase the risk of poor graft function or primary nonfunction and are referred to as "marginal" organs[7]. In this review, we define marginal liver grafts for LDLT as small-for-size grafts, older donors, moderate or severe steatosis of liver grafts, chronic hepatitis, and grafts with tumors. The survival of recipients with marginal organs can be the same as that of patients with high-quality liver grafts under proper treatment[8].

Many previous studies have reported on the possibilities of using marginal grafts in orthotopic liver transplantation (OLT), but there is still a lack of discussion on this topic, especially regarding the feasibility of using marginal grafts in LDLT. Therefore, the present review aimed to summarize and discuss the possibility of expanding the application of marginal grafts in LDLT.

SMALL-FOR-SIZE GRAFTS IN LDLT

Choosing to use a liver graft can be a remarkably complex decision. There is an increasing trend of patients dying while on waiting lists due to the everyday risk of death or serious complications while waiting; this risk must be balanced against the use of a marginal graft, which may not be feasible. Size mismatching between the graft and the recipient is a critical predictor of the so-called "marginal liver grafts" in LDLT recipients. A small-for-size graft has become the main reason for unsuitability for liver donation in some transplantation centers[9]. The most common index with which to evaluate graft size matching is the graft-to-recipient weight ratio (GRWR) or graft volume (GV)/standard liver volume (SLV). The GRWR was first reported to require a safety range of above 1%; otherwise, the rate of graft survival could decrease[10]. With the increased demand for LDLT and the improvement of surgical techniques, however, many expanded-criteria grafts are used. Accordingly, the accepted arbitrary requirement for GRWR was reduced to 0.8%, and the GV/SLV value was 40%[11,12]. As many transplantation centers accumulated experience on small-for-size grafts for LDLT, grafts with a GRWR < 0.8% were used and reported to be as safe as those with a GRWR ≥ 0.8%[13-17]. After challenging the boundary of GRWR = 0.8%, the acceptable minimum GRWR has been continuously lowered. Lee SD *et al*[18] reported that a GRWR as low as 0.7% is safe and that there is no need to modulate portal pressure in adult-to-adult LDLT using the right-lobe in favorable conditions, such as a low Model for End-Stage Liver Disease (MELD) score. Furthermore, Alim A *et al*[19] even suggested that a GRWR as low as 0.6% may be safe if the MELD score is < 20, donor age is < 45, and there is no evidence of liver steatosis in the donor graft during portal inflow modulation performed according to the portal flow. To date, the reported lowest GRWR of grafts that have been successfully used is between 0.40% and 0.46% (Table 1)[20].

Small-for-size syndrome (SFSS), including small-for-size dysfunction (SFSD) and small-for-size nonfunction (SFSNF), is a concerning and life-threatening complication in patients receiving grafts with a GRWR < 0.8%[21,22]. The incidence of SFSS varies from 4.7% to 27.5% in different LT centers[23-30]. Specifically, the syndrome rate can be as high as 50%-75% in left-lobe LDLT or small-for-size grafts group and as low as 8.4% in right-lobe LDLT[31,32]. Graft size is the only independent predictor of SFSS[31]. However, other studies have described that SFSS can occur even in the presence of a normal GRWR[16]. Regardless of the definition used for SFSS, it seems clear that other key factors should be considered in addition to a mismatched graft size. The

Table 1 Recommended minimum graft-to-recipient weight ratio in different studies

Ref.	Recommended minimum GRWR	*n* (small *vs* large)	One-year survival (small *vs* large)	Five-year survival (small *vs* large)	Study type
Kiuchi *et al*[10] (1999)	1%	276 (49 *vs* 215)	61.2% *vs* 92.6%	NS	RS
Lee *et al*[11] (2003)	0.8%	141 (10 *vs* 131)	Univariate and multiple analysis	NS	RS
Moon *et al*[13] (2010)	Less than 0.8%	427 (35 *vs* 392)	87.8% *vs* 90.7%	74.1% *vs* 79.4%	RS
Lan *et al*[14] (2009)	Less than 0.8%	89 (15 *vs* 74)	73.3% *vs* 71.6%	NS	RS
Selzner *et al*[15] (2009)	Less than 0.8%	271 (22 *vs* 249)	91.0% *vs* 89.0%	83.0% *vs* 87%	RS
Chen *et al*[16] (2014)	Less than 0.8%	196 (45 *vs* 151)	82.2% *vs* 81.4%	71.1% *vs* 75.5%	RS
She *et al*[17] (2017)	Left lobe graft *vs* right lobe graft	218 (19 *vs* 199)	89.5% *vs* 95.9%	89.5% *vs* 86.8%	RS
Lee *et al*[18] (2014)	Less than 0.7%	317 (23 *vs* 294)	100% *vs* 93.2%	NS	RS
Alim *et al*[19] (2016)	0.6%	649	Seven patients had GRWR of 0.6%. If MELD score was below 20, donor age below 45, and no signs for any hepatosteatosis, GRWR of 0.6% was safe		RS
Lee *et al*[20] (2015)	0.40%	NS	Lowest GRWR of 0.40% had been successfully used		RS

PS: Prospective study; RS: Retrospective study; Ref.: Reference; GRWR: Graft-to-recipient weight ratio.

Table 2 Incidence of small-for-size syndrome when using small-for-size grafts *n* (%)

Ref.	*n*	SFSS (Incidence)	Factors to SFSS	Study type
Goldaracena *et al*[21] (2017)	NS	NS	A graft GRWR < 0.8% of predisposes the graft to SFSS	RE
Graham *et al*[22] (2014)	NS	NS	GRWR of 0.8 to 1.0 was established as a lower limit to prevent SFSS	RE
Botha *et al*[23] (2010)	21	1 (4.7)	Hemi-portocaval shunt can decrease SFSS incidence	RS
Goralczyk *et al*[24] (2011)	22	5 (22.7)	Posterior cavoplasty can decrease SFSS incidence	RS
Soejima *et al*[25] (2003)	36	8 (22.2)	Cirrhosis predisposes the graft to SFSS	RS
Ben-Haim *et al*[26] (2001)	40	5 (8)	Child's class B or C with received grafts of GRWR < 0.85% predisposes the graft to SFSS	RS
Sudhindran *et al*[27] (2012)	NS	10%-20%	Left lobe grafts predisposes the graft to SFSS	RE
Yi *et al*[28] (2008)	29	8 (27.5)	Left lobe grafts predisposes the graft to SFSS	RS
Soejima *et al*[29] (2012)	312	43 (15.3)	Left lobe grafts predisposes the graft to SFSS	RS
Gruttadauria *et al*[30] (2015)	83	13 (15.7)	Non-surgical modulation of the portal inflow can decrease SFSS incidence	RS
Shoreem *et al*[31] (2017)	174	20 (11.5)	Left lobe grafts predisposes the graft to SFSS	RS
Lauro *et al*[32] (2007)	8	4 (50)	Surgical modulation of the portal inflow can decrease SFSS incidence	RS

RE: Review; RS: Retrospective study; SFSS: Small-for-size syndrome; GRWR: Graft-to-recipient weight ratio.

incidence of SFSS is listed in Table 2.

Middle hepatic vein (MHV) or outflow reconstruction of the liver graft is associated with size mismatch. A small-for-size graft without MHV reconstruction can lead to various degrees of congestion of the anterior segment and a greater loss of hepatocellular function[33]. In our early observational studies with small sample sizes, we recommended a GRWR > 1.0%[34] or even 1.2%[35] as a security threshold for patients without MHV reconstruction. Asakuma M *et al*[36] established an algorithm known as the estimated congestion ratio (ECR, ECR = regional volume of v5 + v8 / right-lobe volume) to estimate whether MHV should be reconstructed for low-GRWR grafts. A liver with an ECR > 0.4 is an MHV-dominant liver, and higher GRWR grafts should be used. However, it is still unknown how far we can lower the GRWR following the improvement of postoperative management and surgical technique if there is no reconstruction of outflow. In addition to outflow reconstruction, the inflow of grafts, including portal hypertension following reperfusion and the hyperdynamic splanchnic state, is reported as a major factor that can trigger SFSS[37-39]; however, these views are controversial[40]. Enhanced cholestasis, hepatocyte ballooning, disruption of the sinusoidal line, and transformation of activated Ito cells into fibroblasts are observed under the conditions of portal hypertension, or overperfusion[41,42]. Recipients with a final portal vein pressure (PVP) ≤ 15 mmHg or a pressure gradient of PVP-central vein pressure (CVP) ≤ 5 mmHg have a better prognosis[43]. In another study, liver-graft-to-spleen-volume ratio was used to predict early graft function in children and young adults undergoing LDLT, in which < 0.88 predicted portal hyperperfusion[44]. Moreover, a MELD score > 20[45], a decline in the platelet (PLT) count at post operation day (POD) 3 > 56%[46] and donor age > 45 years are also risk factors for a poor prognosis in recipients of small-for-size grafts[19].

To increase the safety of the expanded use of small-for-size grafts, some treatments are recommended. Graft inflow or PVP modulation is at the forefront of these treatments. Portosystemic shunting techniques

Table 3 Remedies when using small-for-size graft

Ref.	*n*	Remedy for using small-for-size graft	Study type
Botha *et al*[23] (2010)	21	Hemi-portocaval shunt can decrease SFSS incidence	RS
Goralczyk *et al*[24] (2011)	22	Posterior cavoplasty can decrease SFSS incidence	RS
Kim *et al*[47] (2017)	160	Preserving collateral veins on small-for-size grafts	RS + PSM
Hessheimer *et al*[48] (2011)	NS	Portocaval shunt	AE
Xiao *et al*[49] (2012)	1	Transjugular intrahepatic portosystemic shunt	CR
Sato *et al*[50] (2008)	4	Portocaval shunt using ligamentum teres	CR
Nutu *et al*[51] (2018)	2	Complete splenic embolization	CR
Badawy *et al*[52] (2017)	164	Splenectomy	RS
Troisi *et al*[53] (2016)	NS	Splenic artery ligation, splenectomy, meso-caval shunt, spleno-renal shunt, portocaval shunt, and splenic artery embolization	SR
Xu *et al*[54] (2015)	NS	Dual grafts	RE
Gao *et al*[55] (2017)	NS	Adipose-derived mesenchymal stem cells tranplantation	AE
Kobayashi *et al*[56] (2009)	5	Auxiliary partial liver transplantation	CR

PSM: Propensity score matching; AE: Animal experiments; CR: Case report; SR: Systematic review; RE: Review; SFSS: Small-for-size syndrome.

or preservation of collateral veins[19,47-50], as well as splenectomy or splenic artery ligation/embolization[51-53], are effective ways to address post-transplantation portal hyperperfusion. In cases where the GRWR of grafts is very low, dual grafts can be considered[54]. Moreover, autologous stem cell implantation[55] and auxiliary partial LDLT (a second transplant) are also reported to treat SSFS[56]. Remedies when using small-for-size grafts are listed in Table 3.

Generally, a GRWR < 0.8% is no longer a critical predictor for recipients and can even be lowered to 0.5%-0.6% if there are accompanying factors of PVP ≤ 15 mmHg, MHV reconstruction, or young donor age.

OLDER DONORS IN LDLT

Because LDLT allows more choices in the use of a suitable liver graft compared with OLT, elderly donors were rarely considered in the early years of transplantation. However, following the increasing demands for LDLT and the urgent need to save the lives of patients suffering from hepatic encephalopathy, the use of elderly liver grafts has been reported more frequently in recent years as a means to increase the donor pool and address high waiting list mortality[57]. In Japan, the percentages of donors older than 50 and 60 years were 18.1% and 4%, respectively[58]. It is expected that the number of older donors will increase in the future because of the continuing donor shortage[59].

The definition of older donors is quite different in different transplantation centers. In the present review, we define older donors as donors older than 50 years. Controversy exists regarding the use of livers from older donors. The liver regeneration rate is impaired in older donors (donor age ≥ 50 years) compared with young donors (donor age < 30 years), according to computed tomography (CT) volumetric data after LDLT at POD 7[60], and donor age (≥50 years) was independently correlated with impaired remnant liver regeneration at 3[61] and 6 mo in right-lobe LDLT[58]. Kawano Y *et al*[62] analyzed telomeres in the hepatocytes of 12 paired donor-recipients and found that donor age was a crucial factor affecting the sustainability of telomere length in hepatocytes after pediatric LDLT. Based on the conclusion that older donors were significantly associated with impaired liver regeneration, some researchers found that the recipients of grafts from donors older than 45-50 years old, along with a GW/SLV ratio < 35%-40%, had worse outcomes[63,64]. Yoshizumi T *et al*[65] established the following formula, called a predictive score, to evaluate the impact of donor age, graft size, and MELD score on prognosis: predictive score = 0.011 × graft weight (%) - 0.016 × donor age - 0.008 × MELD score - 0.15 × shunt (if present) - 1.757. Patients with a predictive score ≥ 1.3 had a lower incidence of postoperative complications and a better prognosis.

Additionally, more studies have shown that LDLT using older donors could induce more serious postoperative complications and higher mortality rates than transplants using younger donors[66-70]; similarly, having a donor older than the recipient by > 20 years is problematic[68]. Moreover, it has been reported that fibrosis progression in patients with recurrent hepatitis C tended to be faster after LDLT with grafts from older donors[71]. Donor age is an independent, strong prognostic factor in LDLT. However, other researchers found that grafts from older donors can be used safely, even though the regenerative capacity of older grafts is impaired when the donor age is ≥ 50 years[72-75] or even ≥ 55 years[76]. The impact of older donors on the 1- and 5-year survival of recipients is shown in Table 4.

While donor age is a controversial topic, the impaired regenerative capacity of older grafts has been confirmed in some studies. According to these previous studies, older liver grafts can be prudent candidates but cannot be used in the presence of other marginal conditions (*e.g.,* small-for-size grafts or moderate and severe steatosis). More high-quality and prospective studies are needed on this topic.

Table 4 Older donors for living donor liver transplantation

Ref.	Definition of older donors	*n* (older *vs* young)	One-year survival (older *vs* young)	Five-year survival (older *vs* young)	Study type
Tanemura *et al*[58] (2012)	50 yr old	101 (24 *vs* 77)	Older donor livers might have impaired regenerative ability		RS
Ono *et al*[60] (2011)	50 yr old	15 (6 *vs* 9)	Liver regeneration is impaired with age after donor hepatectomy		RS
Akamatsu *et al*[61] (2007)	50 yr old	299 (62 *vs* 237)	85.0% *vs* 93.0%	72.0% *vs* 87.0%	RS
Kawano *et al*[62] (2014)	NS	12	Donor age is a crucial factor affecting telomere length sustainability in hepatocytes after pediatric LDLT		PS
Imamura *et al*[63] (2017)	NS	198	A worse outcome might be associated with aging of the donor		RS
Dayangac *et al*[64] (2011)	50 yr old	150 (28 *vs* 122)	78.6% *vs* 83.4%	NS	RS
Yoshizumi *et al*[65] (2008)	NS	28	Graft size, donor age, and patient status are the indicators of early graft function		RS
Han *et al*[66] (2014)	55 yr old	604 (26 *vs* 578)	Median OS (M): 31.2 ± 31.3 *vs* 50.6 ± 40.6		RS
Kamo *et al*[67] (2015)	60 yr old	1597 (69 *vs* 1528)	69.5% *vs* 81.2%	62.0% *vs* 79.3%	RS
Shin *et al*[68] (2013)	Donor-recipient age gradient > 20	821	Worse graft survival was observed if the donor is older than the recipient by > 20		RS
Kubota *et al*[69] (2017)	50 yr old	315 (126*vs* 189)	73.0% *vs* 80.9%	39.7% *vs* 47.1%	RS
Katsuragawa *et al*[70]	NS	24	G/SLV and donor age were independent factors that affected graft survival rates		RS
Wang *et al*[72] (2015)	50 yr old	159 (10 *vs* 149)	100% *vs* 93.0%	90.0% *vs* 87.0%	RS
Ikegami *et al*[73] (2008)	50 yr old	232 (32 *vs* 200)	80.0% *vs* 81.7%	73.8% *vs* 76.7%	RS
Li *et al*[74] (2012)	50 yr old	129 (21 *vs* 108)	90.0% *vs* 86.0%	66.0% *vs* 75%	RS
Goldaracena *et al*[75] (2016)	50 yr old	469 (91 *vs* 378)	92.0% *vs* 96.0%	83.0% *vs* 79.0%	RS
Kim *et al*[76] (2017)	55 yr old	540 (42 *vs* 498)	95.2% *vs* 94.6%	NS	RS

LDLT: Living donor liver transplantation; CR: Case report; RS: Retrospective study.

ABO-INCOMPATIBLE LDLT

Although more high-quality liver grafts are available for patients in LDLT than in OLT, donors are restricted to family members or domestic relationships in many transplantation centers because of ethical norms. ABO-incompatible LTs are performed only in emergencies, when ABO-compatible grafts are unavailable. Therefore, breaking ABO blood group barriers becomes inevitable. ABO-incompatible LT was first performed and reported by Starzl *et al*[77], and no acute rejections were observed after transplantation. Subsequently, ABO-incompatible LT gradually began to be performed in some LT centers, and hyperacute rejection was commonly reported[78,79].

In addition to antibody-mediated rejection, ABO-incompatible LDLT can involve other complications. Thrombotic microangiopathy (TMA) is a rare complication following LT, but it is reported to have a slightly higher incidence in ABO-incompatible LDLT[80-82]. ABO incompatibility, cyclophosphamide and recipient blood group (type O) are closely correlated with the occurrence of TMA[80,82]. The incidence of TMA is 37.9% following ABO-incompatible LDLT and 0.0%-2.8% following ABO-compatible LDLT (OR = 44.7)[80]. The elevation of fibrinolytic function markers, such as plasminogen activator inhibitor type 1, can be considered a predictor of TMA following LDLT. The incidence of biliary tract complications is more common than that of TMA. Biliary strictures are one of the most important complications associated with ABO incompatibility, with reported incidence rates between 15.8% and 20.7%[83,84]. An isoagglutinin attack on the graft vascular endothelium can result in ischemic cholangiopathy, and isoagglutinin can even directly attack the endothelium of the graft bile duct[85,86]. CT scans can provide a clear indication of biliary strictures in ABO-incompatible LDLT[87]. Yamada Y *et al*[88] reported a case of idiopathic hypereosinophilic syndrome following ABO-incompatible LDLT. The patient suffered from portal vein thrombosis on postoperative day 10, and the histopathological findings of the thrombus revealed dense eosinophilic deposition. Studies on the impact of ABO incompatibility on LDLT are listed in Table 5.

Despite serious complications, ABO-incompatible LDLT can be a feasible option for patients if certain essential treatments are included[89,90]. Rituximab, an anti-CD20 immunoglobulin (IgG)1 terminating B-lymphocytes with an affinity for IgG Fc receptor (FcγR), is a critical strategy in the regimens for desensitization for ABO-incompatible LDLT and yields outcomes for ABO-incompatible LDLT that are similar to those for ABO-compatible LDLT[91,92]. Rituximab is given for 3 d[93], 3 wk, or even as soon as a suitable donor that is ABO-compatible is selected[94] at a dosage of 375 mg/m^2. In the early stage of transplantation, rituximab was usually given along with one or more other protocols, such as a splenectomy[95,96], plasma exchanges[97-102], intravenous IgG[100,103], and intrahepatic arterial infusion of prostaglandin E1[92,104,105]. In some recent studies, pre-transplant rituximab and/or basiliximab monotherapy, without additional treatments, also yielded outcomes that are comparable to those of procedures with additional treatments[106]. The affinity between IgG Fcγ Receptor (FcγR) and rituximab, however, is influenced by the single-nucleotide polymorphisms (SNPs) of FcγR. SNPs of FCGR2A (131H/R) and FCGR3A (158F/V) are the alleles that encode FcγR. FCGR2A (131H/H) had a higher affinity for IgG1 than FCGR2A (131H/R

Table 5 Impact of ABO-incompatible on living donor liver transplantation

Ref.	*n*	Complications	Incidence of related complication (%)	Risk factors	Study type
Miyata *et al*[80] (2007)	57	Thrombotic microangiopathy	7.0	ABO-incompatibility, CPA, and recipient blood group (type O)	RS
Oya *et al*[81] (2008)	1	Thrombotic microangiopathy	NS	ABO-incompatible LDLT (type B to O)	CR
Kishida *et al*[82] (2016)	129	Thrombotic microangiopathy	10.1	ABO-incompatible, tacrolimus	RS
Song *et al*[83] (2014)	1102	Biliary stricture	15.8	ABO-incompatible, acute cellular rejection	RS
Ikegami *et al*[84] (2016)	408	Biliary stricture	20.4	ABO-incompatible	RS
Yamada *et al*[88] (2010)	1	Idiopathic hypereosinophilic	NS	ABO-incompatible	CR

LDLT: Living donor liver transplantation; CR: Case report; RS: Retrospective study.

or R/R). Accordingly, patients with FCGR2A (131H/H) have a better reaction to the effects of rituximab on B cells[91]. The treatment results of ABO-compatible LDLT are summarized in Table 6.

These findings reveal that rituximab monotherapy in ABO-compatible LDLT is feasible, but it is better to test the SNPs of FcγR; otherwise, multiple treatments, such as plasma exchanges and intravenous IgG, must be performed in addition to rituximab if there is a lower affinity between IgG FcγR and rituximab. There is still a lack of more persuasive evidence to confirm the feasibility of splenectomy in conjunction with ABO-compatible LDLT treatments.

LIVER GRAFT STEATOSIS

Steatosis is a common feature used to identify marginal liver function, and reports on the utility of steatotic liver grafts in clinical practice have yielded controversial results. The use of steatotic liver grafts has been confirmed to have a significant relationship with increased complications and poorer outcomes[107,108]. Traditionally, steatotic livers with > 60% fat must be discarded. Livers with < 30% fat are feasible and anticipated to have good function. Livers with 30%-60% fat have poor results, with decreased graft survival and decreased patient survival[109]. Moreover, hepatic steatosis is reported to be a leading cause of donor rejection in LDLT[110]. In some transplantation centers, approximately 40% of donor grafts are discarded because of severe liver steatosis[9]. Because of the release of inflammatory cytokines and inhibition of the capacity to differentiate steatosis hepatocytes, the early regenerative capacity of the remnant liver is injured, and, as a result of impaired hepatocyte replication, compensatory expansion of hepatic progenitor cells occurs during steatotic liver regeneration after LDLT[111]. Furthermore, Cho *et al*[112] confirmed that hepatic steatosis is associated with intrahepatic cholestasis and transient hyperbilirubinemia during regeneration after LDLT. In this study, 67 LDLT recipients examined on POD 10 were scored based on the numbers of portal tracts per area of liver tissue and intrahepatic cholestasis, and the preoperative degree of macrovesicular steatosis was found to be independently associated with cholestasis after LDLT. However, these researchers also found that the long-term capacity of hepatocyte regeneration was not impaired after LDLT with mild macrovesicular steatosis grafts[113]. Based on this finding, some recent studies have found that moderately steatotic liver grafts and donors with a BMI ≥ 30 kg/m^2 are not contraindications for LDLT, and complications and survival are not significantly different compared with those associated with non-steatosis grafts[114,115]. Moreover, the risk of steatosis was determined by the presence of microsteatosis and macrosteatosis, rather than the total quantitative degree of steatosis. The grafts with high microsteatosis (30%) mixed with macrosteatosis showed no significant difference in postoperative biochemical liver function, 2-wk graft regeneration, postoperative complications, and 5-year survival[116]. The studies on the impact of graft steatosis on LDLT outcomes are listed in Table 7.

To decrease the risk associated with fatty liver grafts, especially with severe steatosis, some treatments are suggested (Table 8). According to Oshita *et al*[117], donors who are diagnosed with hepatic steatosis pre-transplantation should undergo a diet treatment consisting of an 800-1400 kcal/d diet and a 100-400 kcal/d exercise regimen without drug treatment with a target body mass index of 22 kg/m². After these strategies, the average BMI was reduced from 23.3 ± 0.6 to 21.9 ± 0.4 kg/m². The liver biopsy results of most of these donors showed stage 0/1 fibrosis and minimal/mild steatosis after the diet therapy. In addition, surgical outcomes and overall survival did not significantly differ between the recipients of grafts from non-steatosis and diet-treated donors (with steatosis). In another study, bezafibrate (400 mg/d) was used along with a protein-rich (1000 kcal/d) diet and exercise (600 kcal/d) for 2-8

Table 6 Remedies when using ABO- incompatible on living donor liver transplantation

Ref.	*n*	Immunosuppression strategy	Remedies	Conclusion	Study type
Kawagishi *et al*[89] (2009)	105	TAC + MP + AZ	Rituximab	ABO-incompatible LDLT can be feasible used if humoral rejection are overcome	RS
Yoon *et al*[90] (2018)	918	TAC + MP + steroids	Rituximab and PE	ABO-incompatible LDLT is a feasible option under remedies	RS
Sakai *et al*[91] (2017)	20	TAC+ MP	Rituximab and PE	FCGR SNPs influence the effect of rituximab on B-cells	PS
Egawa *et al*[92] (2017)	33	TAC	Rituximab, PE, local infusion, splenectomy and immunoglobulins	Only rituximab dose is a significantly favorable factor for AMR	RS
Ikegami *et al*[93] (2007)	1	TAC + MP + steroids	Rituximab and PE	Rituximab and plasma exchanges seemed ineffective	CR
Ikegami *et al*[94] (2009)	7	TAC + MP + steroids	Rituximab, IVIG, and PE	Rituximab, IVIG, and PE seems to be a safe treatment	RS
Usui *et al*[95] (2007)	73	TAC + MP + steroids	Rituximab, PE and splenectomy	Bone suppression is a big challenge when using rituximab	RS
Chen *et al*[96] (2017)	2	TAC + MP + steroids	Basiliximab combine with splenectomy	ABO-i LDLT with splenectomy is undoubtedly life-saving	CR
Uchiyama *et al*[97] (2011)	15	TAC + MP + steroids	Rituximab and PE	Isoagglutinin mediated-rejection should be more concerned	RS
Soin *et al*[98] (2014)	3	TAC + MP + steroids	Rituximab and PE	ABO-incompatible LDLT is a feasible option under remedies	CR
Rummler *et al*[99] (2017)	10	TAC + MP + steroids	PE	Immunosuppression only combining with PE is feasible	RS
Kim *et al*[100] (2016)	182	TAC + MP + steroids	Rituximab, IVIG, and PE	ABO-incompatible LDLT can be safely performed under remedies	RS
Kim *et al*[101] (2013)	22	TAC + MP + steroids	Rituximab and PE	ABO-incompatible LDLT can be safely performed under remedies	RS
Kawagishi *et al*[102] (2005)	3	TAC + MP + steroids	Rituximab and PE	ABO-incompatible LDLT can be safely performed under remedies	CR
Kim *et al*[103] (2017)	43	TAC + MP + steroids	Rituximab and IVIG	A simplified protocol using rituximab and IVIG for ABO-I LDLT is safe	RS
Yoshizawa *et al*[104] (2005)	8	TAC + MP + cyclophosphamide	Rituximab and PGE1 infusion	Rituximab prophylaxis and HA infusion therapy is feasible	RS
Egawa *et al*[105] (2008)	118	TAC + steroids	Methylprednisolone and PGE1 infusion	Recipients with preexisting high effector CD8 T- cells are unfavorable candidates for ABO-I LDLT	RS
Yamamoto *et al*[106] (2018)	40	TAC + MP + steroids	Rituximab monotherapy	Rituximab monotherapy is feasible	RS

LDLT: Living donor liver transplantation; CR: Case report; RS: Retrospective study; SNPs: Single-nucleotide polymorphisms.

wk[118]. Even severely steatotic livers could be used for LDLT grafting subsequent to this short-term treatment regimen. Furthermore, a 1200 kcal/d diet and a minimum of 60 min/d of moderate cardio training are also recommended to rapidly reverse liver steatosis in donors[119]. In addition to lifestyle and dietary changes, dual-graft LDLT was reported when one donor had severe liver steatosis and another had a low GRWR[120].

In conclusion, steatosis in the donor must be thoroughly evaluated before LDLT, either by biopsy or imaging diagnosis. The proportion of macrosteatosis is now considered a crucial predictor of the prognosis of recipients. If there are no further options, donors with hepatic steatosis can reach donation criteria through lifestyle and dietary changes in a short time.

CHRONIC HEPATITIS OF GRAFTS

The use of liver grafts that test positive for chronic hepatitis or other blood disseminated diseases found in epidemic areas is usually inevitable in cases of organ shortages associated with OLT. However, because LDLT recipients, to some degree, have more choices regarding his/her donors, there are a few studies reporting on HBsAg or HBcAb(+) liver grafts, while no studies refer to HCV-positive living liver grafts.

HBsAg(-) LDLT patients who have received HBsAg or HBcAb(+) grafts have a high risk of *de novo* HBV

Table 7 Impact of graft steatosis on living donor liver transplantation

Ref.	*n*	Conclusion	Study type
Dirican *et al*[9] (2015)	161	Approximately 40% of donor grafts are discarded because of severe liver steatosis	RS
Perkins *et al*[109] (2006)	NS	Typically steatotic livers with > 60% fat are not transplanted; with < 30% fat are usable and anticipated to have good function; with 30%-60% fat give poor results	Comments
Kotecha *et al*[110] (2013)	340	Hepatic steatosis is a leading cause of donor rejection in LDLT	PS
Cho *et a*[111]*l* (2010)	54	Hepatocyte replication is impaired during steatotic liver regeneration after LDLT	PS
Cho *et al*[112] (2006)	67	Hepatic steatosis is associated with intrahepatic cholestasis and transient hyperbilirubinemia during regeneration	PS
Cho *et al*[113] (2005)	55	Mildly steatotic graft did not increase the risk of graft dysfunction or morbidity in LDLT	PS
Gao *et al*[114] (2009)	24	Moderately steatotic (30%-60%) liver grafts provide adequate function in the first phase after transplantation and can be used for transplantation	RS
Knaak *et al*[115] (2017)	105	Donors with BMI > 30, in the absence of graft steatosis, are not contraindicated for LDLT	RS
Han *et al*[116] (2015)	211	The risk of steatosis may be determined by the relative composition of MiS and MaS, rather than the total quantitative degree	RS

LDLT: Living donor liver transplantation; RS: Retrospective study; PS: Prospective study.

Table 8 Treatments for fat donors

Ref.	*n*	Treatments	Study type
Oshita *et al*[117] (2012)	128	Diet treatment consisting of an 800 to 1400 kcal/d diet and a 100 to 400 kcal/d exercise regimen without drug treatment, targeting body mass index of 22 kg/m^2	RS
Nakamuta *et al*[118] (2013)	11	Bezafibrate (400 mg/d) was used along with a protein-rich (1000 kcal/d) diet and exercise (600 kcal/d) for 2-8 wk	RS
Choudhary *et al*[119] (2015)	16	1200 kcal/d and a minimum of 60 min/d of moderate cardio training are also recommended to rapidly reverse liver steatosis in donors	PS
Moon *et al*[120] (2006)	2	Dual-graft living donor liver transplantation for severe graft steatosis	CR

RS: Retrospective study; PS: Prospective study; CR: Case report.

infection after transplantation (Table 9). However, these grafts are still considered to be safe and feasible with antiviral prophylaxis in both adult and pediatric LDLT[121-126]. Patients were given HBV vaccinations to achieve anti-HBs > 1000 IU/L pre-transplantation and > 100 IU/L post-transplantation, with a standard post-transplantation treatment regimen of high-dose hepatitis B IgG, lamivudine and/or adefovir (in cases of lamivudine resistance)[126]. Specifically, some studies have proposed a new strategy; specifically, patients with a pre-transplantation anti-HB titer > 1000 IU/L do not need post-transplantation prophylaxis; patients with a low pre-transplantation titer, < 1000 IU/L, should be given lamivudine post-transplantation (at a dose of 100 mg/d or 3 mg/kg/d for at least 2 years after transplantation) or adefovir prophylaxis (with lamivudine at a dose of 10 mg/d if a mutant strain for lamivudine is identified) and, hopefully, will respond appropriately to post-transplantation vaccinations by maintaining anti-HB titers > 100 IU/L; and low titer non-responders (anti-HB titer < 100 IU/L despite vaccination) should be given continuous lamivudine or adefovir indefinitely[121]. In some transplantation centers, nucleotide analogs (lamivudine) are routinely used first if HBsAg(-) LDLT patients receive HBsAg or HBcAb(+) grafts, regardless of the anti-HB titer, for at least 2 years. Moreover, patients who had a YMDD mutation were given adefovir combined with lamivudine[123]. Hara Y *et al*[127] reported one patient who experienced spontaneous eradication of *de novo* HBV after LDLT with an HBcAb(+) graft without any treatment. This 8-year-old female patient (HBsAg-negative) underwent LDLT, received an HBcAb(+) left-lobe graft, and was subsequently infected with HBV. Sixteen years after LDLT, her serological HBV status was as follows: HbsAg(-), HBsAb(+), HBeAb(-), HBeAb(+), HbcAb(+), and HBV DNA(-). In another study, recipients with HCV genotype 2 infections who had received an HBcAb(+) graft were given sofosbuvir and ribavirin, along with hepatitis B IgG to prevent recurrence of HCV and HBV[128].

In HbsAg(+) LDLT patients who receive HBsAg or HBcAb(+) grafts, the antiviral protocol must be performed as for HBsAg(-) LDLT patients to maintain the HBV DNA at a low or negative level, despite the persistence of the HBV marker (HBsAg). High-dose HBV IgG, lamivudine, famciclovir, and interferon were recommended (Table 10)[129-131].

Populations with HBsAg-negative/HBcAb-positive and undetectable serum HBV DNA have been gradually increasing over the past several decades. Most patients are now considered to have a covert HBV infection and have a high risk of HBV reactivation when treated with

Table 9 Impact of HBsAg or HBcAb(+) grafts on HBsAg(-) living donor liver transplantation patients

Ref.	Donor	Incidence of *de novo* HBV infection (%)	Prevention of *de novo* HBV infection	Study type
Wang[121] (2017)	HBcAb(+)	4.2	HBV vaccinations with the aim of achieving anti-HBs > 1000 IU/L pre-transplant and > 100 IU/L post-transplant	RS
Xi *et al*[122] (2013)	HBcAb(+)	23.9	No prophylaxis, adefovir, and lamivudine are given to *de novo* patients	RS
Dong *et al*[123] (2017)	HBcAb(+)	7.9	HBIG 100 IU/kg during the operation and lamivudine 3 mg/kg per day after the surgery for at least 1 year until HBV vaccine reaction	RS
Loggi *et al*[124] (2016)	HBsAg(+)	NS	HBIG and lamivudine, adefovir or tenofovir	SR
Lei *et al*[125] (2013)	HBcAb(+)	15.0	No specific prophylaxis	RS
Lin *et al*[126] (2007)	HBcAb(+)	3.3	Lamivudine monoprophylaxis, HBV vaccinations	RS
Hara *et al*[127] (2016)	HBcAb(+)	NS	Lamivudine first and adefovir dipivoxil were combined with lamivudine 2 yr later	CR

HBV: Hepatitis B virus; RS: Retrospective study; SR: Systematic review; CR: Case report.

Table 10 Impact of HBsAg or HBcAb(+) grafts on HBsAg(+) living donor liver transplantation patients

Ref.	Donor	Incidence of *de novo* HBV infection	Prevention of De Novo HBV infection	Study type
Hwang *et al*[129] (2006)	HBsAg(+)	NS	High-dose HBIG and lamivudine, famciclovir and interferon; a final regimen of lamivudine and adefovir	CR
Soejima *et al*[130] (2007)	HBsAg(+)	NS	lamivudine and adefovir dipivoxil	CR
Jeng *et al*[131] (2015)	HBsAg(+)	NS	Entecavir 0.5 mg once daily	RS

HBV: Hepatitis B virus; CR: Case report; RS: Retrospective study.

Table 11 Graft with hepatic benign tumor

Ref.	*n*	Type of tumors in grafts	Prognosis	Study type
Li *et al*[133] (2017)	15	Cavernous hemangioma, perivascular epithelioid cell tumor, inflammatory pseudotumor, and focal nodular hyperplasia	One patient died from pulmonary embolism	OS
Fuchino *et al*[134] (2017)	1	HBsAg(+) and inflammatory pseudotumor	Tumor vanished after 3 yr	CR

OS: Observational study; CR: Case report.

a robust immunosuppressive agent[132]. Therefore, the use of HBsAg-negative/HBcAb-positive liver grafts has a high risk of *de novo* HBV for HBsAg(-) recipients. However, with active immunization and an antiviral protocol, the HBsAg-negative/HBcAb-positive liver grafts can be transplanted safely.

GRAFTS WITH A BENIGN HEPATIC TUMOR

Usually, there are rare recipients of LDLT or doctors who are willing to make an active choice to use a graft with an undetermined tumor. This is not only an ethical issue but also indicates a high risk for recipients to face rapid dysfunction of their liver grafts. However, if recipients are in an emergency situation and have no other proper donors, grafts with benign tumors may be a last choice. Li G *et al*[133] recently reported on 15 consecutive recipients using an otherwise discarded, partial liver resection graft with a benign hepatic tumor. These benign tumors are as follows: Cavernous hemangioma, perivascular epithelioid cell tumor, inflammatory pseudotumor, and focal nodular hyperplasia. One patient died from a pulmonary embolism, and the other 14 patients had a good prognosis. Additionally, a vanishing tumor in a liver graft from an HBV(+) donor was observed. Contrast-enhanced magnetic resonance imaging (MRI) showed hypervascularity in the arterial phase and in the hepatobiliary phase, the tumor showed a low intensity, findings similar to those in HCC. Regardless, the graft with suspected HCC was accepted by the recipient, and the tumor disappeared completely within several months after LDLT[134].

For LDLT patients using grafts with a benign hepatic tumor, only two observational studies with a small sample size are present in the literature (Table 11). It seems that grafts with benign tumors are feasible in some conditions, but more studies with long-term follow-ups are needed to evaluate the safety of these

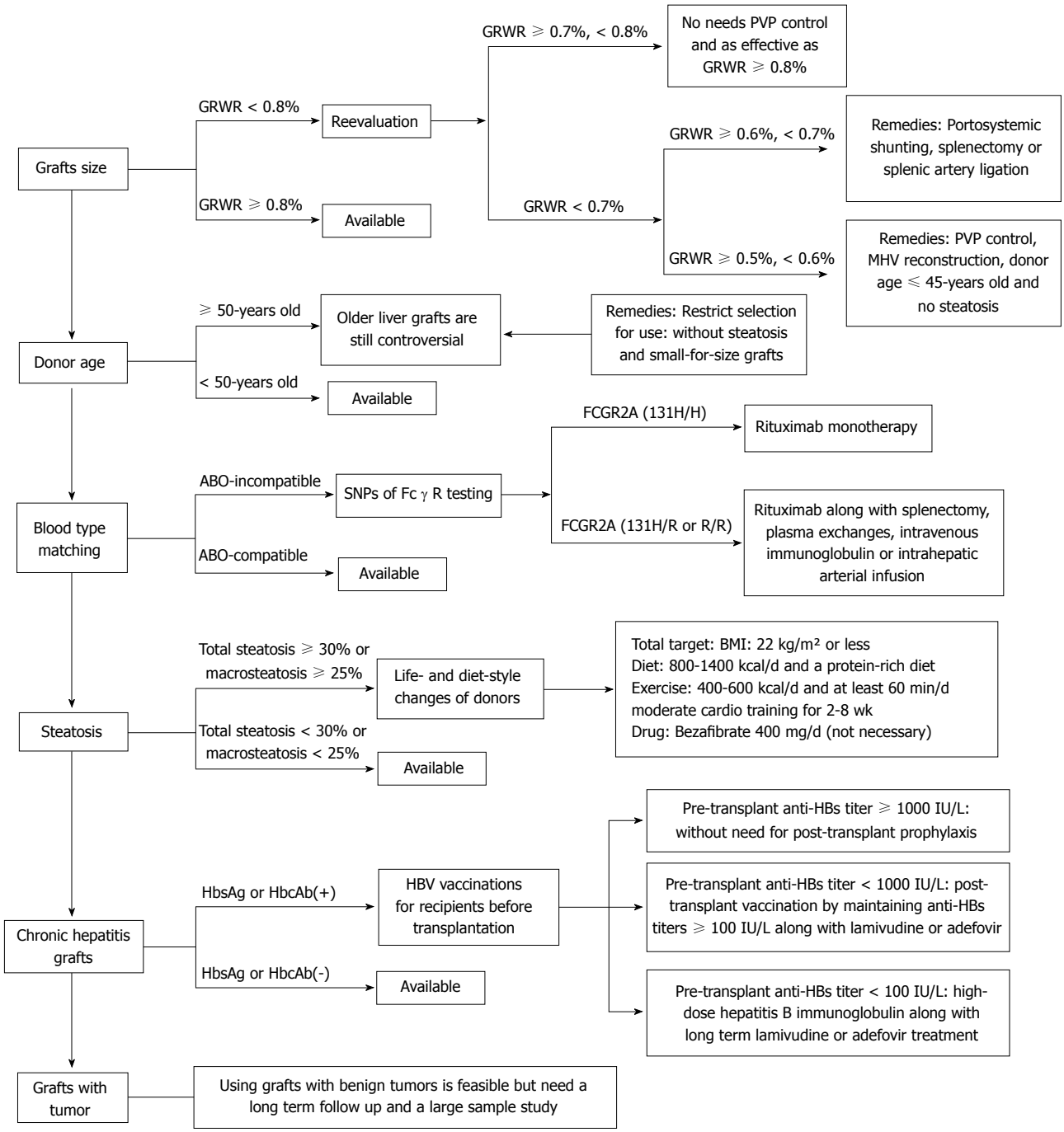

Figure 1 Selective strategies and remedies of using marginal donors in living donor liver transplantation.

marginal grafts.

CONCLUSION

To our knowledge, this is the first review on marginal donors specifically for LDLT. This review, which includes cohort studies, case-control studies, and case reports on marginal liver grafts in LDLT, demonstrated that marginal grafts are a feasible way to expand the options for patients on LT waiting lists in emergency situations (*e.g.*, liver failure or hepatic encephalopathy); however, these grafts place the recipients at a greater risk of liver dysfunction. Some indispensable treatments are needed to address the deficiencies of these grafts.

There are some new findings in this review: (1) It is permissible for the GRWR to be as low as 0.5%-0.6% (not 0.8%, as currently specified) if PVP is controlled under 15 mmHg; otherwise, outflow reconstruction is needed. (2) There is controversy surrounding older liver grafts. These grafts can be used prudently, but other marginal conditions must be absent (*e.g.,* small-for-size grafts or moderate and severe steatosis). (3) Splenectomy is no longer necessary when an ABO-incompatible LDLT is performed. Rituximab monotherapy is even confirmed

to be an effective treatment if there is a high affinity between IgG FcγR and rituximab. (4) Total steatosis of liver grafts is not a proper predictor of prognosis. Instead, the presence of microsteatosis and macrosteatosis is a crucial factor. Donors with steatosis of the liver can meet the donation criteria through lifestyle and dietary changes before surgery. (5) HbsAg or HbcAb(+) grafts increase the risk of *de novo* HBV infection after transplantation in HBsAg(-) LDLT patients but can also be used safely with active immunotherapy. And (6) Grafts with benign tumors that have been discarded from other patients are feasible, but the long-term prognosis cannot be determined.

According to the new findings of this review listed above, we summarized a selection of strategies for different types of marginal liver grafts in LDLT and their related treatments (Figure 1). With this review, based on more than 100 references, we expect that the transplantation pool can be effectively and safely expanded in the situation of organ shortage.

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MINIREVIEWS

Vedolizumab for inflammatory bowel disease: From randomized controlled trials to real-life evidence

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Abstract

The biologic antitumor necrosis factor alpha (anti-TNFα) agents have revolutionised the treatment of inflammatory bowel disease (IBD). However, some patients experience primary nonresponse, loss of response, or intolerance. Therefore, introducing a newer class of therapy with a mechanism of action that acts on different inflammatory pathways involved in IBD pathogenesis is appealing. Vedolizumab is a fully humanised monoclonal antibody that selectively targets α4β7 integrin. Based on the results of the pivotal clinical GEMINI trials, vedolizumab was approved for the treatment of adult patients with moderately to severely active ulcerative colitis (UC) and Crohn's disease (CD) refractory or intolerant to either conventional therapy or TNFα inhibitors. This review describes the efficacy, safety, and tolerability of vedolizumab reported in both randomized, controlled, clinical trials and from real-world experience in patients with UC and CD in order to identify its place in treatment algorithms for IBD.

Key words: Vedolizumab; Crohn's disease; Real-world; Efficacy; Ulcerative colitis; Controlled trial; Effectiveness; Safety

Core tip: Vedolizumab represents an interesting new therapeutic option for the treatment of patients with moderate-to-severe ulcerative colitis and Crohn's disease that are refractory or intolerant to either conventional treatments or anti-TNFα agents. This review describes the efficacy, safety, and tolerability of vedolizumab demonstrated in the clinical GEMINI trials. In addition, the paper reviews the effectiveness and the safety of vedolizumab in the real-world studies in order to identify its place in treatment algorithms for patients with inflammatory bowel disease.

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INTRODUCTION

The introduction of biologic, antitumor necrosis factor alpha (anti-TNFα) therapies has transformed the management of patients with moderate-to-severe, active inflammatory bowel diseases (IBD) that are refractory to conventional treatments[1-3]. However, a proportion of patients do not respond to these drugs, loose their response over time, or are intolerant to these treatments[4-6]. Additionally, the efficacy of a second anti-TNFα agent is lower in patients who have previously received an anti-TNFα drug[7]. Therefore, the advent of a newer class of therapy, characterized by a different mode of action, is an attractive option for patients with IBD.

Vedolizumab is a fully humanised monoclonal IgG-1 antibody that selectively inhibits the interaction between α4β7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). It prevents lymphocyte translocation from the blood into the inflamed gut tissue, resulting in a reduction in local inflammation[8,9] (Figure 1).

The efficacy of vedolizumab for the induction and maintenance of remission in patients with IBD was demonstrated in the pivotal phase III GEMINI studies[10-12]. Based on the results of these randomized, double-blind, placebo-controlled trials, vedolizumab was approved for the treatment of adult patients with moderate-to-severe active ulcerative colitis (UC) and Crohn's disease (CD) who had an inadequate response to either standard therapies or TNFα antagonists by both the European Medicines Agency and the US Food and Drug Administration.

However, all randomized controlled trials (RCTs) have restrictive enrolment criteria and, in order to include a highly selected and homogeneous population, tend to exclude several groups of patients[13]. This limits the generalisation of RCT results to patients commonly seen in general practice.

Patients in real-world cohorts tend to have more complicated diseases, multiple comorbidities, variable treatment regimens applied with flexibility, and follow-up controls that are not fixed. In addition, the goals of therapy in clinical practice are variable and are specific for the single patient ("treat to target")[14]. Therefore, evaluating biologic therapies is highly relevant in the clinical practice setting.

To date, several real-world studies on the effectiveness and safety of vedolizumab in patients with moderate-to-severe, active UC and CD have been published[15-26]. This paper reviews the efficacy of vedolizumab for the treatment of IBD from the randomized controlled clinical trials (GEMINI program) and in the GEMINI long-term safety (LTS) study[27,28], the effectiveness of vedolizumab in the real-world studies, and the drug's safety profile.

EFFICACY OF VEDOLIZUMAB FROM RCTS

Vedolizumab in UC

The efficacy of vedolizumab for inducing and maintaining remission in patients with UC was demonstrated in the GEMINI 1 study, a trial involving more than 800 patients with moderate-to-severe UC, defined as a Mayo score[29] of 6-12, with an endoscopic subscore ≥ 2[10]. The trial consisted of two induction cohorts; a double-blind cohort including 374 patients randomized to receive vedolizumab 300 mg intravenous (iv) or placebo at weeks 0 and 2, and a second additional cohort of 521 patients receiving open-label vedolizumab aimed to generate the needed number of responders to fulfil sample-size requirements for the maintenance phase. Eligible patients had no response to or unacceptable adverse events from steroids, immunosuppressive drugs, or anti-TNFα therapy.

In the first cohort, a significantly higher rate of patients treated with vedolizumab achieved clinical response, clinical remission, and mucosal healing after 6 wk compared to placebo. The primary outcome of the induction phase, clinical response at week 6, was achieved by 47.1% of patients treated with vedolizumab *vs* 25.5% of patients in the placebo group ($P < 0.001$) (Table 1).

Patients from both cohorts achieving clinical response to vedolizumab at 6 wk were randomized to receive vedolizumab 300 mg iv every 4 wk or 8 wk, or to receive placebo in the maintenance phase for up to 52 wk. The results of the maintenance phase were as impressive as those in the induction phase. The rates of clinical remission at week 52, the primary outcome of the maintenance phase, were significantly higher in patients treated with vedolizumab than in those treated with placebo (44.8% in the vedolizumab 4-weekly group, 41.8% in the vedolizumab 8-weekly group, and 15.9% in the placebo group; $P < 0.001$). Durable clinical remission (defined as remission at week 6 and week 52) was also reported by significantly more patients in the vedolizumab groups (24.0% in the vedolizumab 4-weekly group, 20.5% in the vedolizumab 8-weekly group, and 8.7% in the placebo group; $P = 0.001$ and $P = 0.008$, respectively, *vs* placebo). Vedolizumab was also associated with greater mucosal healing rates ($P < 0.001$ for both vedolizumab groups *vs* placebo) and significantly higher rates of steroid-free remission ($P < 0.001$ for both vedolizumab groups *vs* placebo) (Table 1).

A clear difference in efficacy between the 4- and 8-weekly vedolizumab regimens was not observed. Efficacy was reported by both patients with previous exposure to anti-TNFα therapy as well as those who

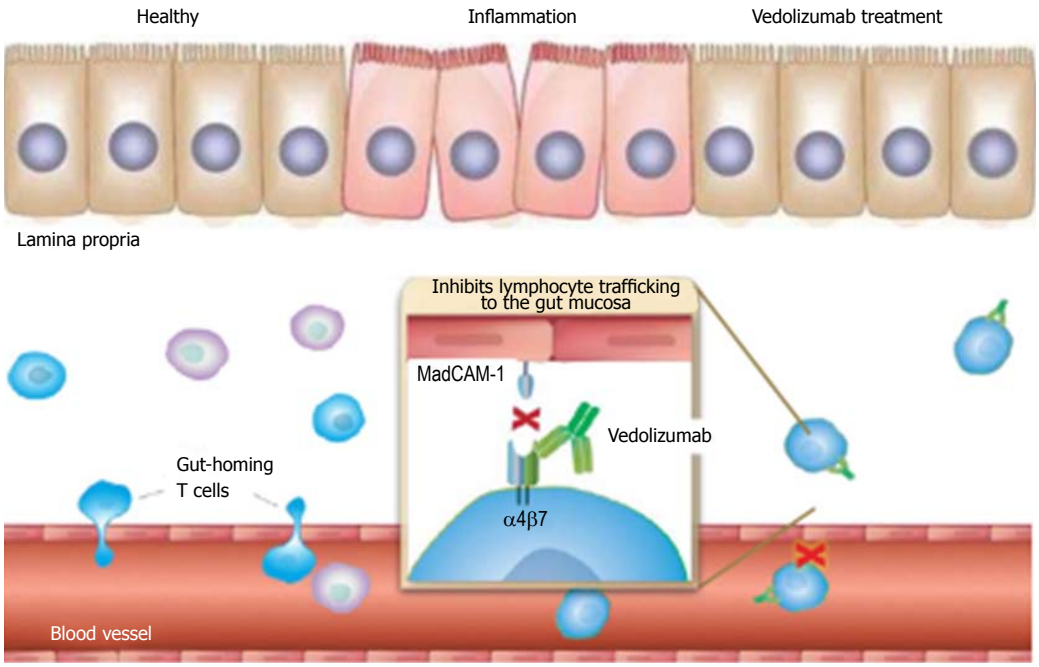

Figure 1 Vedolizumab targets the α4β7 integrin, preventing leucocyte translocation from the blood into the inflamed gut tissue. MadCAM-1: Mucosal addressin cell adhesion molecule.

were anti-TNFα therapy-naïve; however, slightly better outcomes were seen in patients who were TNFα-inhibitor-naïve.

Vedolizumab in CD

The efficacy of vedolizumab in patients with moderately to severely active CD was demonstrated in the GEMINI 2 and GEMINI 3 clinical trials[11,12]. In GEMINI-2, 368 patients were randomized to receive either vedolizumab 300 mg iv or placebo at week 0 and week 2[11]. Additionally, as in the GEMINI 1 trial, a second cohort of 747 subjects was treated with vedolizumab in an open-label fashion. All patients enrolled had active disease defined by a Crohn's Disease Activity Index (CDAI)[30] of 220-450, and had one of the following: serum C-reactive protein (CRP) > 2.87 mg/L or colonoscopic documentation showing ≥ 3 large ulcers or ≥ 10 aphthous ulcers, or faecal calprotectin concentrations > 250 μg/g in conjunction with computed tomography or magnetic resonance enterography, small-bowel radiography, or capsule endoscopy revealing Crohn's ulcers. Eligible patients had no response to or unacceptable adverse events from steroids, immunosuppressive drugs, or anti-TNFα drugs.

Two coprimary endpoints in the induction trial, clinical remission and CDA-100 response, were evaluated at week 6. A significantly greater proportion of patients receiving vedolizumab achieved clinical remission at 6 wk with respect to the placebo group (14.5% *vs* 6.8%; *P* = 0.02) (Table 1). However, the CDAI-100 response rate was comparable to the placebo (31.4% *vs* 25.7%; *P* = 0.23).

During the maintenance phase, 461 patients who were vedolizumab responders were randomized to receive vedolizumab 300 mg iv administered at either 4- or 8-weekly intervals up to week 52. Clinical remission at week 52, the primary endpoint of this phase, was significantly greater in patients assigned to vedolizumab therapy every 4 wk or 8 wk (36.4% and 39.0%) than in the placebo group (21.6%; *P* = 0.004 and *P* < 0.001, respectively, *vs* placebo). The rates of steroid-sparing remission were also significantly higher among patients treated with vedolizumab (*P* = 0.04 and *P* < 0.02, respectively, *vs* placebo), while the rates of durable clinical remission showed no significant differences (Table 1).

Similar results were observed in the GEMINI 3 trial, which evaluated the efficacy of vedolizumab in 315 patients with moderately to severely active CD and inadequate response, loss of response, or intolerance to previous TNFα antagonists[12]. Patients were assigned randomly to receive vedolizumab 300 mg iv or placebo at weeks 0, 2, and 6. Clinical remission at week 6 was observed in 15.2% of vedolizumab patients compared to 12.1% in the placebo group (*P* = 0.4) (Table 1). Therefore, the primary endpoint of the study was not met. However, the rates of clinical remission at week 10 were significantly higher in patients treated with vedolizumab (26.6% *vs* 12.1% in the placebo group; p = 0.001). The benefit in this population was therefore observed at week 10, suggesting a delayed response in obtaining clinical remission. In clinical practice, there is an opportunity for a fourth induction dose at week 10 in patients with CD, with insufficient response to the first three administrations of vedolizumab.

A meta-analysis pooling data from the phase II

Table 1 Phase III randomized controlled trials of vedolizumab in patients with ulcerative colitis and Crohn's disease

Study	*n* (patients)	Setting of trial	Treatment arms	Clinical response (%)	Clinical remission (%)	CS-free remission (%)	Mucosal healing (%)
GEMINI 1[10] 2013	374	Induction	300 mg	47.1	16.9	-	40.9
			Placebo	25.5	5.4		24.8
		Maintenance	300 mg 4 weekly	-	44.8	45.2	56.0
			300 mg 8 weekly		41.8	31.4	51.6
			Placebo		15.9	13.9	19.8
GEMINI 2[11] 2013	368	Induction	300 mg	31.4	14.5	-	-
			Placebo	25.7	6.8		
		Maintenance	300 mg 4 weekly	45.5	36.4	28.8	-
			300 mg 8 weekly	43.5	39.0	31.7	
			Placebo	30.1	21.6	15.9	
GEMINI 3[12] 2015	315	Induction	300 mg	-	15.2	-	-
			Placebo		12.1		

Clinical response was defined as a reduction in the Mayo score of at least 3 points plus a decrease of at least 30% from the baseline score, with a decrease in the rectal bleeding subscore ≥ 1, an absolute rectal bleeding subscore ≤ 1 (GEMINI 1), or as a ≥ 100-point decrease in the CDAI score (GEMINI 2). Clinical remission defined as a Mayo score of ≤ 2 and no subscore > 1 (GEMINI 1) or as a CDAI score ≤ 150 points (GEMINI 2, GEMINI 3). CS: Corticosteroid.

and phase III randomized controlled studies involving patients with active CD showed that vedolizumab increased the rates of clinical remission and CDAI-100 response during the induction phase, although it failed to meet some of the primary endpoints of the GEMINI 2 and GEMINI 3 trials[31].

LONG-TERM EFFICACY OF VEDOLIZUMAB IN IBD FROM CLINICAL TRIALS

An interim analysis of the efficacy data from the GEMINI LTS study was recently published[27,28]. The GEMINI LTS study is an ongoing, open-label, extension trial in patients with UC and CD designed to investigate the long-term safety of vedolizumab in patients with IBD. In addition, an exploratory evaluation of long-term clinical efficacy was also performed. Patients were enrolled from the long-term, phase II, C13004 study and from the GEMINI 1, GEMINI 2, and GEMINI 3 trials. A remaining part of the population consisted of patients with IBD who were vedolizumab-naïve who were included directly into the GEMINI LTS trial. A total of 894 patients with UC and 1349 with CD were enrolled in the GEMINI LTS. All patients received vedolizumab 300 mg iv every 4 wk.

Populations evaluated during the efficacy analysis of the GEMINI LTS included only patients with moderate-to-severe UC (532/894) or CD (1297/1349); patients from the C13004 study were excluded because some patients with mild IBD were enrolled in this study.

Outcomes of clinical response and remission, evaluated using a partial Mayo score in UC and the Harvey-Bradshaw Index[32] in CD, were assessed after up to 152 wk of therapy. The results showed that among patients with UC having a response to vedolizumab at week 6 in the GEMINI 1 study, 88% (*n* = 120/136) and 96% (*n* = 70/73) were in clinical remission after 104 and 152 wk, respectively. Similarly, the rates of remission reported by the patients with CD who responded to the induction phase of the GEMINI 2 study were 83% (*n* = 100/120) and 89% (*n* = 62/70) at the same time points.

An increase in dosing frequency in patients who had withdrawn early from the GEMINI 1 and GEMINI 2 studies, treated every 8 wk to every 4 wk in the GEMINI LTS trial, resulted in remission rates of 28% and 32%, respectively, after 52 wk. Similar improvements were observed regardless of previous anti-TNFα therapy.

A retrospective evaluation of mucosal healing after treatment with vedolizumab in patients with IBD enrolled in the GEMINI LTS study at Leuven University Hospital was recently reported[33]. A total of 58 patients (34 UC, 24 CD), previously exposed to anti-TNFα therapy, were endoscopically followed for a median duration of 3.2 years. Mucosal healing, corrected with non-responder imputation, was reported by 50% of patients with UC and 29% with CD. Additionally, 32.4% of patients with UC and 20.8% with CD achieved histological healing. A significant correlation between mucosal and histological healing was observed in both patients with UC and CD.

EFFECTIVENESS OF VEDOLIZUMAB IN PATIENTS WITH IBD FROM REAL-WORLD STUDIES

Several prospective and retrospective real-life studies of vedolizumab in patients with moderate-to-severe UC and CD have been published by authors from Europe and the United States[15-26].

European real-life studies

To date, the French GETAID group (Groupe d' Etude Therapeutique des Affections Inflammatoires du tube Digestif) has published the largest real-world cohort

Table 2 Real-world studies on vedolizumab in patients with inflammatory bowel disease, Crohn's disease, or ulcerative colitis

Study author (country) years	IBD	CD	UC
Amiot *et al*[15,16] (France), 2016-2017	294	173	121
Eriksson *et al*[19] (Sweden), 2017	246	147	99[1]
Baumgart *et al*[17] (Germany), 2016	212	97	115
Dulai *et al*[23] (United States, multicentre), 2016	212	212	-
Kopylov *et al*[20] (Israel), 2017	204	130	74[1]
Shelton *et al*[24] (United States, Boston), 2015	172	107	65[1]
Macaluso *et al*[22] (Italy, Sicily), 2018	163	84	79
Allegretti *et al*[26] (United States, Boston), 2017	136	96	40
Stallmach *et al*[18] (German Registry), 2016	127	67	60
Vivio *et al*[25] (United States, Saint Louis), 2016	102 (51)[2]	30	21
Samaan *et al*[21] (United Kingdom), 2017	50	27	23[1]
Total	1.918	1.170	697

[1]UC + IBD unclassified; [2]102 patients started vedolizumab, and 51 patients were followed prospectively. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

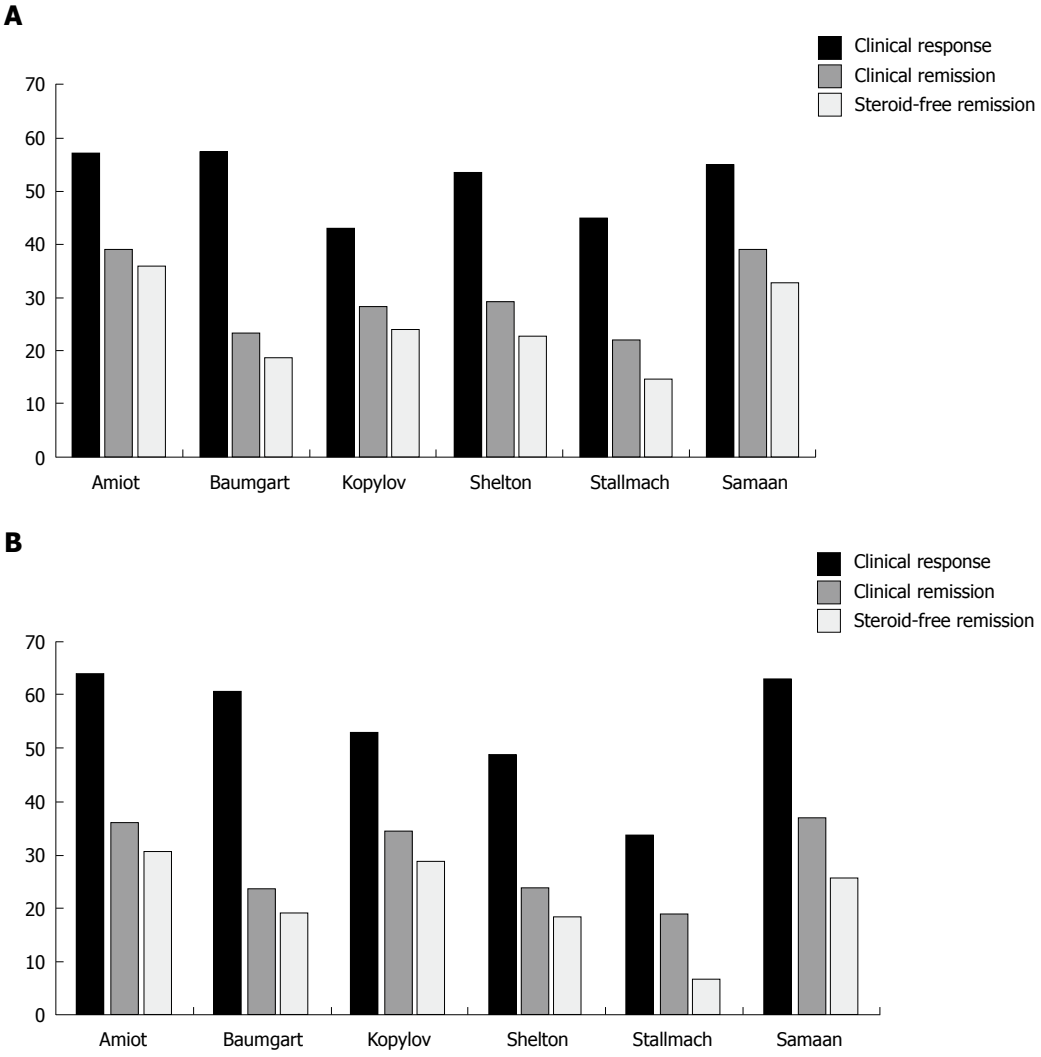

Figure 2 Real-world studies with vedolizumab in patients with ulcerative colitis (A) and Crohn's disease (B): Results at week 14.

comprising 294 patients with moderately to severely active IBD who were followed prospectively until week 54[15,16] (Table 2). Almost all patients had previously failed at least one anti-TNFα agent, with 91% having failed two. Patients received vedolizumab 300 mg iv at weeks 0, 2, and 6 then every 8 wk afterward up to week 52. At week 6, 32% of patients with UC and 31% with CD were in clinical remission. The primary outcome of the induction study, steroid-free clinical remission at week 14, was reported by 36% and 31%

of patients with UC and CD, respectively[15] (Figure 2A and B). At week 54, steroid-free clinical remission rates were 40.5% in patients with UC and 27.2% in patients with CD[16]. Mucosal healing, assessed between weeks 30 and 54, occurred in 54.8% of patients with UC and 29.8% with CD. However, it was evaluated in only a small proportion of the population, and it is possible that patients with active disease were reassessed less frequently. A significant number of patients experienced inadequate response or loss of response during the year of treatment, and vedolizumab therapy was optimized (300 mg every 4 wk) in 54% of patients. Dose optimization induced or restored clinical response in 41% of patients, of whom 30% achieved clinical remission.

Predictors of clinical effectiveness were assessed by the authors, who found that a clinical response at week 6, baseline CRP > 20 mg/L, and a higher baseline disease activity were predictive of steroid-free remission at week 14 in both groups[15]. In addition, patients with UC and CD who achieved a clinical response at week 6 were more likely to achieve steroid-free clinical remission at week 54 ($P < 0.001$)[16].

A German National cohort study prospectively included 212 patients with IBD, most of whom were anti-TNFα experienced[17] (Table 2). The results showed that clinical remission at week 6 was reached by 11.3% and 15.5%, and at week 14 by 23.5% and 23.7%, of patients with UC and CD, respectively (Figure 2A and B). This group identified a low HBI at baseline and hospitalization in the past 12 mo as independent predictors of clinical remission at week 14 in patients with CD.

These data were followed by a longer-term study that included some patients from the previous German induction cohort as well as additional patients with IBD[18] (Table 2). Based on nonresponding imputation analysis, clinical remission was observed in 22% and 19% of patients with UC and CD, respectively, at week 14 (Figure 2A and B). Clinical remission at week 54 was reported by 15/60 (25%) patients with UC and 14/67 (21%) patients with CD. Nonresponse status at week 14 was an indicator of a low likelihood of clinical response and remission at week 54 in patients with both diseases. In addition, the reduction of CRP at week 14 in patients with UC and CD, and of faecal calprotectin in patients with UC, was predictive of clinical remission at week 54.

More recently, a large prospective cohort based on the Swedish National Quality Registry for IBD (SWIBREG), evaluating the data at week 12, 52 and the last follow-up, reported that clinical remission was obtained with vedolizumab after 52 wk in 64% and 60% of patients with UC and CD, respectively, 86% of whom had previously used TNFα inhibitors[19] (Table 2). Elevated CRP at baseline and prior use of anti-TNFα were associated with a higher risk of vedolizumab discontinuation.

The large, Israeli, real-world study in patients with IBD who had high rates of previous anti-TNFα therapy, showed similar efficacy of vedolizumab in patients with UC and CD[20] (Table 2; Figure 2A and B). A retrospective cohort of 50 patients with IBD from the UK demonstrated similar rates of effectiveness compared to the other real-world studies[21] (Table 2; Figure 2A and B).

Very recently, real-world data on the effectiveness of vedolizumab on gut and articular symptoms in 163 patients with IBD were reported by an Italian group[22] (Table 2). Steroid-free remission was observed in 71 (43.6%; UC: 45.6%, CD: 41.7%) and 29 (40.8%; UC: 40.0 %, CD: 41.7%) patients at weeks 10 and 22, respectively. At the same time points, a response on articular symptoms was achieved in 39.5% and 45.4% of patients with IBD who had active spondyloarthritis at baseline. The only factor associated with response on articular manifestations was the coexistence of a concomitant intestinal benefit at both weeks 10 and 22. These data suggest that the improvement of articular symptoms could be mainly related to the intestinal response.

United States real-world studies

A large real-world cohort in the United States was published by the US VICTORY (Vedolizumab for Health Outcomes in Inflammatory Bowel Diseases) consortium and included 212 patients with CD, 90% of whom were TNFα-antagonist exposed[23] (Table 2). This retrospective study of seven medical centres from across the United States reported rates of clinical remission of 18%, 35%, and 54% at 6, 12 and 18 mo, respectively. Prior TNFα-inhibitor use, severe disease activity, active perianal disease, and smoking history were associated with a lower likelihood of achieving clinical remission. Cumulative rates of mucosal healing and "deep remission" (defined as a combination of clinical remission and mucosal healing) after 12 mo were observed in 63% and 26% of patients with CD, respectively. Patients with previous anti-TNFα treatment and severe disease activity were less likely to obtain mucosal healing.

In another study from the United States, two centres in Boston enrolled 172 patients with IBD, almost all with previous use of TNFα antagonist[24] (Table 2). Similar rates of clinical response, clinical remission, and steroid-free remission at week 14 were reported by patients with UC and CD (Figure 2A and B). Early response at week 6 was a significant predictive factor of week 14 response/remission in patients with UC, with a trend toward significance in those with CD. Elevated CRP (> 8 mg/L) at baseline was associated with a lower likelihood of achieving clinical response/remission in patients with both diseases.

Vivio *et al*[25] reported data on 102 patients with IBD, of whom 51 were followed prospectively (Table 2). At week 14, 55% of the patients with UC in the

prospective cohort achieved clinical remission. Rates of mucosal healing and endoscopic improvement after a median treatment duration of 22 wk were higher in patients with UC (69% and 76%) than in patients with CD (30% and 52%).

COMBINATION OF VEDOLIZUMAB AND ANTI-TNFα AGENTS IN THE TREATMENT OF IBD

The combination of vedolizumab and anti-TNFα drugs (infliximab or adalimumab) in the treatment of IBD was very recently reported by a case series of 6 patients with UC and 4 patients with CD[34]. Before combination therapy, all patients were treated with anti-TNFα, however they still had an active disease even after the optimization of the dosage and/or the infusion interval. At the time of inclusion 4 patients received concomitant immunomodulators and 1 patient received systemic corticosteroid. The patients were prospectively followed for at least 12 mo (median 17 mo) and at the end of the follow-up period all patients achieved clinical remission, and 8 out of 10 could stop the anti-TNFα treatment.

In 2 case reports previously published vedolizumab was successfully used in association with an anti-TNFα drug for the treatment of a patient with chronic refractory pouchitis and axial spondylarthritis and a patient with CD and erythema nodosum[35,36].

These data suggest that combination treatment of vedolizumab and anti-TNFα therapy might represent a therapeutic option in selected patients with IBD, however further larger studies are needed.

PREDICTORS OF RESPONSE TO VEDOLIZUMAB

Several factors have been evaluated as predictors of response and remission to vedolizumab and a recent review summarized the current data[37]. Overall, patients with less disease activity (by clinical and inflammatory indices), naïve to TNFα inhibitors, and having higher vedolizumab trough levels at induction[38-40] had a greater likelihood of responding to treatment in both disease groups.

Concomitant immunomodulatory treatment was not associated with improved results in the GEMINI 1 and 2 studies[10,11]. However, the interpretation of these data is limited by the small sample size of patients receiving concomitant immunomodulators and their interruption during the maintenance period in both trials. In addition, the studies were not designed to address outcomes in patients on combination immunosuppressive therapy. No consistent benefit of adding an immunomodulatory agent to vedolizumab was observed in some real-world studies and in a small group of patients with IBD[15,20,23,24,41]. These data are in line with the finding that combination therapy did not lead to higher early vedolizumab trough levels[38]. A potential explanation is the low immunogenic profile of vedolizumab, which differs from that of anti-TNFα agents[42,43]. Currently, only a multicentre study and a recent case series showed that the addition of an immunomodulatory agent to vedolizumab was associated with an increased clinical response and remission in patients with CD and UC[26,44]. Further studies are needed to better define this aspect.

SAFETY OF VEDOLIZUMAB FROM RANDOMIZED CONTROLLED TRIALS AND REAL-WORLD STUDIES

Safety data on vedolizumab have been evaluated from four double-blind and two open-label trials in an analysis that included over 2800 vedolizumab-exposed patients with IBD who were treated for up to 5 years[45]. A very good safety profile and minimal immunogenicity were reported.

The risk of progressive multifocal leukoencephalopathy (PML) is a potential safety concern for drugs that block lymphocyte migration. More than 500 cases have occurred in natalizumab-treated patients[46]. However, no cases of PML have been observed during treatment with vedolizumab according to the concept that the gut selectivity of vedolizumab is protective against the development of PML[47].

Vedolizumab is not associated with an increased risk of serious or opportunistic infections, and few patients (< 1%) discontinued therapy because of infection. The most common events were upper respiratory tract infections, which accounted for approximately half of the total infections. Lower respiratory tract, lung infections, and abdominal and enteric infections were reported with similar rates as those in the placebo group. Serious infections including sepsis, *Clostridium difficile* infections, and tuberculosis occurred very rarely (≤ 0.6% of patients). Independent risk factors for serious infections were younger age (HR = 0.97) and concomitant steroid use (HR = 1.88) in patients with CD, and prior anti-TNFα failure (HR = 1.99) in patients with UC. Concomitant narcotic analgesic use was a risk factor for patients with both CD and UC (HR = 2.72 and HR = 2.68, respectively).

The rate of malignancy was consistent with that generally reported in patients with IBD.

A total of 23 hepatobiliary events were observed among vedolizumab-treated patients, most of which were hepatic steatosis and transaminase increases. Five hepatic events were considered serious and vedolizumab was interrupted. Appropriate treatment resulted in resolution or near resolution of the liver abnormalities.

Infusion-related reactions occurred in ≤ 5% of

patients, and < 1% of patients discontinued the infusion or received an incomplete dose. Transient anti-drug antibodies were reported by 4% of subjects enrolled in the GEMINI 1 and GEMINI 2 trials, suggesting that loss of response related to the development of anti-drug antibodies may be a rare event.

Additionally, a post-hoc analyses of the GEMINI 1 and GEMINI 2 studies did not report any significant differences in infections or other adverse events amongst the age groups, confirming a good safety profile in older (> 55 years) patients[48].

No higher rate of adverse events than the one expected with anti-TNFα therapy alone was observed in the small series of patients treated with a combination of vedolizumab and anti-TNFα agents.

The good safety profile of vedolizumab may be related to its mechanism of action, which is characterized by a gut-selectivity, without systemic action.

Cumulative evidence from real-world studies has not pointed out any relevant differences in infectious and noninfectious adverse events compared to those seen in the RCTs[49]. Therefore, postmarketing data have confirmed the favourable safety profile of vedolizumab observed in the GEMINI program.

CONCLUSION

Vedolizumab represents an interesting new therapeutic option for the treatment of patients with UC and CD that are refractory to either conventional treatments or TNFα inhibitors[50]. The efficacy and safety of vedolizumab in patients with IBD were demonstrated in the pivotal GEMINI studies. However, the stringent and restrictive inclusion and exclusion criteria in the study designs may limit the translation of clinical trial results into patients commonly seen in the clinic. In fact, patients enrolled in RCTs only partially represent the IBD population encountered during routine clinical practice[13].

Real-world studies confirm the effectiveness of vedolizumab in the clinical practice setting and have also evaluated long-term data. Even though the interpretation of the data is limited by significant heterogeneity in the study designs, real-world experience series provide additional relevant evidence[51]. A systematic review and pooled analysis on the effectiveness and safety of real-world studies has recently been published[52].

Mucosal healing is a relevant therapeutic target in patients with IBD because it is associated with a reduction in hospitalization, IBD-related surgery, bowel damage, and risk of colonic dysplasia. There is increasing evidence that achieving mucosal healing can favourably alter the natural course of IBD[53,54]. Therefore, the rates of long-term mucosal healing with vedolizumab reported by Noman, in keeping with the one-year mucosal healing data observed in the GEMINI 1 trial and in the real-world US VICTORY consortium study, appear very promising.

Safety data from all the GEMINI studies showed an overall rate of adverse events similar to that reported in the placebo group. In addition, an increasing amount of real-world data has confirmed the reassuring safety profile of vedolizumab over an extended treatment period.

In conclusion, vedolizumab has demonstrated efficacy and safety in patients who failed TNF-α antagonists, and should therefore be considered a valid second-line induction and maintenance therapy for these patients. In addition, along with other biologic drugs, vedolizumab should be considered as a first-line treatment for steroid-dependent and steroid-refractory patients and for patients not responding or intolerant to immunosuppressant agents, thanks to its favourable safety profile. Although head-to-head prospective trials to compare the safety of biologic drugs are not available, in patients in whom it is preferable to avoid systemic immunosuppression (patients with high risk of opportunistic infections or the elderly[55,56]), vedolizumab may be a safer alternative.

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ORIGINAL ARTICLE

Basic Study

Dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient diet-induced nonalcoholic steatohepatitis

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Abstract

AIM

To investigate changes in gut microbiota and metabolism during nonalcoholic steatohepatitis (NASH) development in mice fed a methionine-choline-deficient (MCD) diet.

METHODS

Twenty-four male C57BL/6J mice were equally divided into four groups and fed a methionine-choline-sufficient diet for 2 wk (Control 2w group, n = 6) or 4 wk (Control 4w group, n = 6) or the MCD diet for 2 wk (MCD 2w group, n = 6) or 4 wk (MCD 4w group, n = 6). Liver injury, fibrosis, and intestinal barrier function were evaluated after 2 and 4 wk of feeding. The fecal microbiome and metabolome were studied using 16s rRNA deep sequencing and gas chromatography-mass spectrometry.

RESULTS

The mice fed the MCD diet presented with simple hepatic steatosis and slight intestinal barrier deterioration after 2 wk. After 4 wk of feeding with the MCD diet, however, the mice developed prominent NASH with liver fibrosis, and the intestinal barrier was more impaired. Compared with the control diet, the MCD diet induced gradual gut microbiota dysbiosis, as evidenced by a marked decrease in the abundance of *Alistipes* and the (*Eubacterium*) *coprostanoligenes* group (P < 0.001 and P < 0.05, respectively) and a significant increase in Ruminococcaceae UCG 014 abundance (P < 0.05) after 2 wk. At 4 wk, the MCD diet significantly reduced the promising probiotic *Bifidobacterium* levels and markedly promoted *Bacteroides* abundance (P < 0.05, and P < 0.01, respectively). The fecal metabolomic profile was also substantially altered by the MCD diet: At 2 wk, arachidic acid, hexadecane, palmitic acid, and tetracosane were selected as potential biomarkers that were significantly different in the corresponding control group, and at 4 wk, cholic acid, cholesterol, arachidic acid, tetracosane, and stearic acid were selected.

CONCLUSION

The MCD diet induced persistent alterations in the gut microbiota and metabolome.

Key words: Nonalcoholic steatohepatitis; Methionine-choline deficient diet; Gut microbiota; Metabolome; Nonalcoholic fatty liver disease

Core tip: Nonalcoholic steatohepatitis (NASH) is increasingly prevalent as a remarkable problem worldwide. Increased evidence indicates the critical role of gut microbiota in NASH progression. We aimed to investigate the dynamic alterations in the gut microbiota and the related metabolites during NASH development in mice fed a methionine-choline-deficient (MCD) diet. We for the first time find that the MCD diet may induce persistent gut microbiota and metabolome deterioration.

Ye JZ, Li YT, Wu WR, Shi D, Fang DQ, Yang LY, Bian XY, Wu JJ, Wang Q, Jiang XW, Peng CG, Ye WC, Xia PC, Li LJ. Dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient diet-induced nonalcoholic steatohepatitis. *World J Gastroenterol* 2018; 24(23): 2468-2481 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2468.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2468

INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease worldwide, is increasing, encompassing a pathological spectrum from simple steatosis to nonalcoholic steatohepatitis (NASH)[1]. NASH is a liver characteristic of metabolic syndrome, accounting for lipid accumulation and hepatic inflammation[2] and is projected to be the main indication for liver transplantation in the next decade[3]. NASH is commonly associated with type 2 diabetes, cardiovascular disease, and end-stage kidney disease[4,5], and no approved pharmacological therapies currently exist for NASH[6].

Recently, compelling evidence has indicated the critical role of the gut microbiota in the pathogenesis and progression of NAFLD to NASH[7]. Normally, the commensal gut microbiota and the host maintain a beneficial symbiotic relationship. The liver may function as a vascular firewall, mediating mutualism between the host and the intestinal commensal bacteria[8]. This intimate connection between the gastrointestinal tract and liver defines the term gut-liver axis. However, gut microbiota dysbiosis increases the influx of harmful substances, such as lipopolysaccharide (LPS), ethanol, and bacterial DNA, into the liver through portal vein circulation and promotes NASH development[9,10]. Currently, early and noninvasive diagnosis of NASH with high sensitivity and specificity remains challenging. Metabolomics, along with other "omics" technologies, helps provide a detailed understanding of biochemical events using the systems biology approach and facilitates early diagnosis and exploitation of treatment strategies[11].

Despite the importance of the gut microbiota and metabolome in NASH, detailed information regarding their role during NASH development is limited. The

purpose of the present study was to investigate dynamic alterations in the gut microbiota and metabolome during the development of methionine-choline-deficient (MCD) diet-induced NASH in a mouse model.

MATERIALS AND METHODS

Animals and treatments

The animal protocol was designed to minimize the animals' pain or discomfort and was approved by the Animal Care Committee of Zhejiang University School of Medicine (Permit number: 2017-591). Male C57BL/6J mice (8 wk old) were purchased from SLAC Laboratory (Shanghai, China). After their arrival, the mice were acclimatized for 1 wk in a specific pathogen-free environment (23 ℃, 12 h/12 h light/dark, 50% humidity, and *ad libitum* access to food and water) prior to experimentation. The mice were fed a methionine-choline-sufficient diet (Research Diet, New Brunswick, United States) for 2 wk (the Control 2w group, n = 6) or 4 wk (the Control 4w group, n = 6). Alternatively, the mice were fed an MCD diet (Research Diet) for 2 wk (the MCD 2w group, n = 6) or 4 wk (the MCD 4w group, n = 6). The diet specifications are summarized in Supplementary Table 1. After 2 or 4 wk on the diet, the mice were euthanized by an intraperitoneal injection of 4% chloral hydrate (with 1 mg/100 mL of atropine, to inhibit respiratory secretions) for tissue collection.

Sample collection

Fecal samples were collected from all mice upon defecation and were stored at -80 ℃ for further procedures. Blood samples were centrifuged (3000 rpm, 15 min) for serum separation, and all serum aliquots were stored in a -80 ℃ freezer. Liver and colon samples were fixed in either neutral-buffered formalin or optimal cutting temperature (OCT)-embedding media for histological staining or were snap-frozen in liquid nitrogen and kept in a -80 ℃ freezer for further procedures.

Histopathological evaluation of liver tissue

Paraffin-embedded liver sections were stained with hematoxylin-eosin (HE) or Masson's trichrome to detect liver injury or fibrosis. The stained sections were scanned using a NanoZoomer Digital Pathology system (Hamamatsu Photonics, KK, Japan), which digitally scanned the sections into a particular image format for further assessment. The HE-stained sections were scored in accordance with the NAFLD activity score (NAS) system (score 0-2: Not NASH, 3-4: Borderline NASH, and 5-8: NASH)[12]. Six fields from each section were selected and analyzed at 200 × magnification. The Masson's trichrome-stained sections were analyzed to quantify fibrosis using Image-Pro Plus software (version 6.0, Media Cybernetics, Rockville, United States) as previously detailed[13]. For each section, the blue area (collagen) was normalized to the red area (hepatocyte). The fibrosis index (%) was calculated as a percent of the total tissue region and represented the average of six randomly selected fields from each section.

Oil red O staining and liver triglyceride assay

Frozen liver sections fixed in OCT (10 μm) were stained with Oil red O (Sigma-Aldrich, St. Louis, MO, United States). Images were captured using the abovementioned NanoZoomer Digital Pathology system. To quantify intrahepatic lipid accumulation, the mean optical density of the red intensity was assessed using Image-Pro Plus software.

The liver triglyceride (TG) content was determined using a commercial kit from Applygen Technologies Inc. (Beijing, China) according to the manufacturer's protocols, and the final TG concentrations were normalized to the corresponding protein content.

Immunohistochemistry and immunofluorescence staining

Paraffin-embedded liver sections were stained for F4/80 (anti-active macrophage) (Abcam, Cambridge, United Kingdom) and α-SMA (fibrosis hallmark) with immunohistochemistry (IHC) staining procedures as previously detailed[14]. Briefly, liver sections were incubated with a specific primary antibody, followed by incubation with horseradish peroxidase (HRP)-linked secondary antibody (Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine; the sections were then scanned with the NanoZoomer Digital Pathology system. Image-Pro Plus software was used to count F4/80+ cells and quantitatively analyze the staining intensity of α-SMA as previously described[13]. Six fields of view were randomly selected in each section.

Likewise, paraffin-embedded colon sections were stained for Zonula occludens-1 (ZO-1) (intestinal barrier hallmark) (Proteintech, Rosemont, IL, United States) with standard immunofluorescence staining procedures as previously detailed[15]. Briefly, sections were incubated with the rabbit polyclonal ZO-1 antibody, followed by incubation with Texas Red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, United States) and 4',6-diamino-2-phenyl indole (DAPI), and images were captured using a Zeiss LSM T-PMT confocal microscope (Zeiss, Jena, Germany).

Microbial community analysis of fecal samples

Total DNA was extracted from fecal samples with a QIAamp fast DNA stool mini kit (Qiagen, Valencia, CA, United States) following the manufacturer's handbook. After determining the DNA concentration and integrity, an amplicon sequencing library was constructed based on the PCR-amplified V3-V4 variable regions of 16s rDNA. Then, the qualified libraries were paired-end sequenced on an Illumina MiSeq platform according to manufacturer's procedures. Raw sequencing data were subjected to filtration using Trimmomatic, FLASH, and QIIME software. Then, clean reads were clustered into operational taxonomic units (OTUs) using UPARSE

Table 1 Effects of the methionine-choline-deficient diet on nonalcoholic fatty liver disease activity score, alanine aminotransferase, aspartate aminotransferase, and hepatic triglycerides levels

	Control 2w	MCD 2w	Control 4w	MCD 4w
NAS	0.17 ± 0.17	1.17 ± 0.17[a]	0.33 ± 0.21	4.83 ± 0.31[c,f,j]
Steatosis	0.00 ± 0.00	1.17 ± 0.17[b]	0.33 ± 0.21	2.50 ± 0.22[c,e,j]
Inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.17 ± 0.17[b,e,i]
Ballooning	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	1.17 ± 0.31
TG (nmol/mg protein)	179.41 ± 21.72	592.88 ± 55.84[b]	204.45 ± 6.76[e]	731.60 ± 113.46[a,h]
ALT (U/L)	19.33 ± 0.88	413.83 ± 81.77[a]	34.83 ± 2.64[b,d]	974.17 ± 163.00[a,h]
AST (U/L)	105.50 ± 10.55	327.33 ± 39.92[a]	109.83 ± 11.91[d]	749.677 ± 92.34[b,d,i]

[a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$ *vs* Control 2w group, [d]$P < 0.05$, [e]$P < 0.01$ and [f]$P < 0.001$ *vs* MCD 2w group, [h]$P < 0.05$, [i]$P < 0.01$ and [j]$P < 0.001$ *vs* Control 4w group. The data are shown as the means ± SEM of 6 mice per group. MCD: Methionine-choline-deficient; NAS: NAFLD activity score; NAFLD: Nonalcoholic fatty liver disease; TG: Triglycerides; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

software with a 97% threshold. The representative read from each OTU was selected using the QIIME package[16]. Representative OTU sequences were annotated and taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2, trained on the Silva database version 123[17]. The linear discriminant analysis (LDA) effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/galaxy/) was applied to differentiate taxa with statistical significance and biological relevance[18].

Metabolomic profiling analysis of fecal samples

Metabolomic profiling analysis was performed as previously described[19] with slight modification. Briefly, fecal metabolites were extracted by mixing 15 mg of feces with 800 μL of ice-cold methanol. After homogenization and centrifugation, the supernatant was transferred into an Eppendorf tube containing 20 μL of 1 mg/mL heptadecanoic acid as the internal standard. Then, the sample was dried using a nitrogen stream (Aosheng, Hangzhou, China). The residue was reconstituted in 50 μL of 15 mg/mL methoxylamine hydrochloride in anhydrous pyridine and was incubated at 37 ℃ for 24 h. Then, 50 μL of N,O-bistrifluoroacetamide (BSTFA) [with 1% trimethylsilyl chloride (TMCS)] (Sigma-Aldrich, St. Louis, MO, United States) was added to the mixture, and the sample was incubated at 70 ℃ for 120 min. Metabolomic analysis was performed with a gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890A GC system coupled to an Agilent 5975C inert mass selective detector (MSD) system (Agilent Technologies, Santa Clara, CA, United States). For data analysis, ChemStation software (version E.02.02.1431), and ChromaTOF software (version 4.34, LECO, St. Joseph, MI, United States) were used. Metabolites were identified by the National Institute of Standards and Technology (NIST) and Fiehn databases. Principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed to visualize metabolic differences among the experimental groups. Differential metabolites were selected according to the statistically significant VIP values obtained from the OPLS-DA model, and the *P* values from two-tailed Student's *t*-tests on the normalized peak areas; metabolites with VIP values > 1 and *P* values < 0.05 were included.

Statistical analysis

The data are shown as the means ± SEM, and the Kolmogorov-Smirnov test was performed to assess data normality. For most data, one-way ANOVA with Tukey's post hoc test was used to determine the significance between the groups. The Wilcoxon rank sum test was used to evaluate alpha diversity and principal coordinates between the different cohorts in the 16s sequencing analysis. Analysis of similarities (ANOSIM) was performed to test for microbial community clustering according to unweighted UniFrac distance matrices. *P* values < 0.05 were considered significant. The data were analyzed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

MCD diet resulted in gradual liver injury and intestinal barrier impairment

After 2 wk, the mice fed the MCD diet developed simple hepatic steatosis: the liver showed predominantly microvesicular fat accumulation without lobular inflammation and ballooning (Figure 1A, Table 1). Hepatic steatosis was significantly increased in the MCD 2w group compared with that in the Control 2w group, as further indicated by Oil red O staining and hepatic TG quantification (Figure 1B and D, Table 1). No significant difference was found in the number of F4/80+ cells between the MCD 2w group and the Control 2w group (Figure 1C and E). Compared with the Control 2w group, the MCD 2w group presented significantly increased serum alanine aminotransferase (ALT) ($P < 0.01$) and aspartate aminotransferase (AST) ($P < 0.01$) levels (Table 1). Evidence of liver fibrosis was not found, as shown by Masson's trichrome and α-SMA immunohistochemical staining (Figure 2). Intestinal barrier destruction is associated with NAFLD progression[10]; therefore, we detected tight junction protein expression in the colon, where the most abundant gut microbiota reside[20]. ZO-1 immunostaining revealed that the colon tissues of the mice in the MCD 2w group exhibited

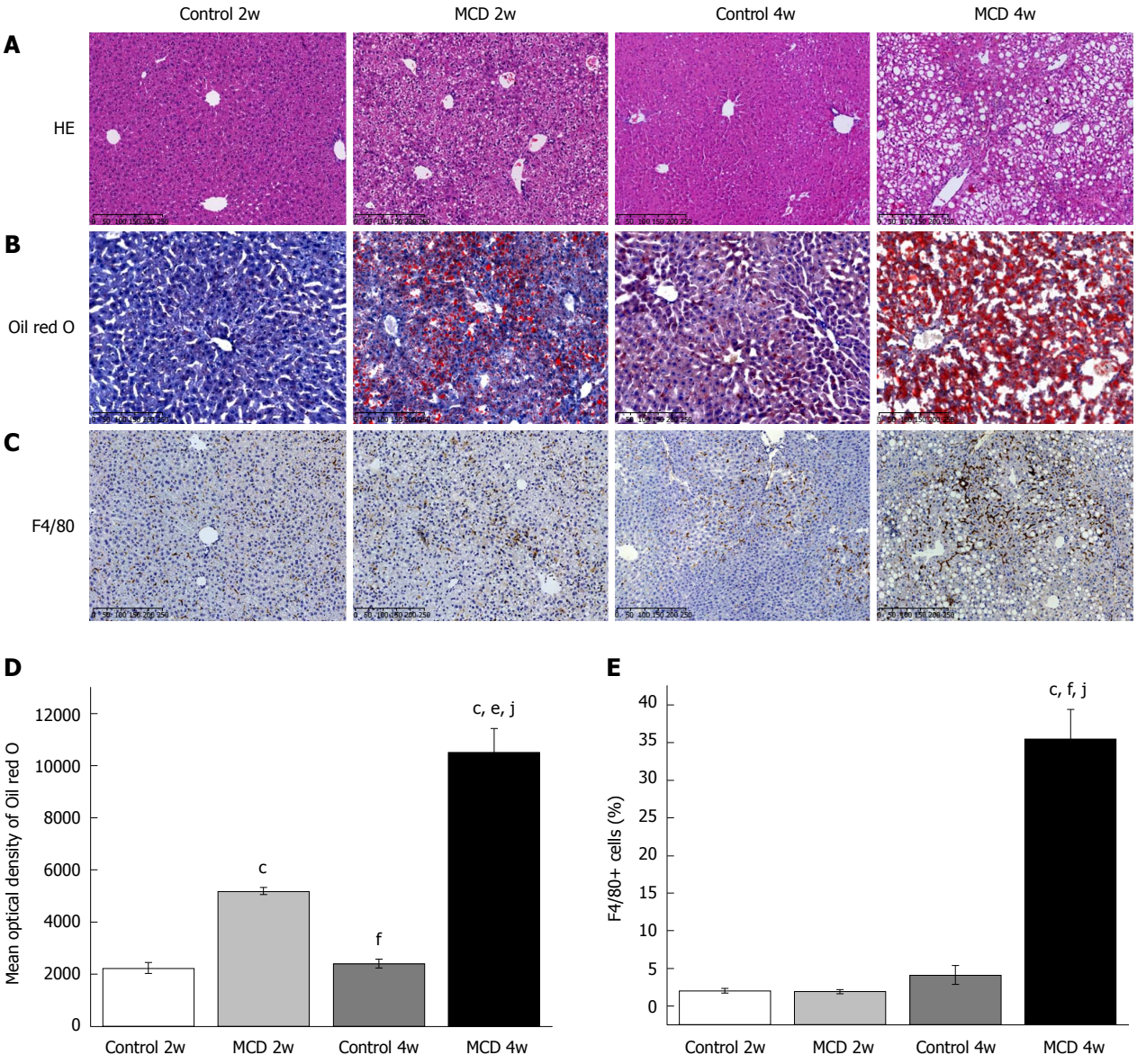


Figure 1 Methionine-choline-deficient diet induced hepatic injury, fat accumulation, and macrophage infiltration. A-C: Representative liver histology assessed by HE staining (A), Oil red O staining (B), and macrophages (F4/80) staining (C). Scale bar: 250 μm. D: Representative staining intensities of Oil red O as designated by mean optical density. E: Percentage of F4/80 positive cells. The data are given as the means ± SEM. n = 6 per group. $^{c}P < 0.001$ *vs* Control 2w group, $^{e}P < 0.01$ and $^{f}P < 0.001$ *vs* MCD 2w group, $^{j}P < 0.001$ *vs* Control 4w group by post hoc ANOVA one-way statistical analysis. MCD: Methionine-choline-deficient.

increased disruption and disorganization on the apical surface and in the crypts (Figure 3).

As expected, the mice in the MCD 4w group developed prominent NASH, as evidenced by major hepatic steatosis with lobular inflammation and ballooning hepatocytes in the liver (Figure 1A, Table 1). Oil red O staining and hepatic TG quantification also revealed that fat accumulation was significantly increased in the livers of the mice fed the MCD diet for 4 wk (the MCD 4w group) compared with that in the mice fed the control diet for 4 wk (the Control 4w group) (Figure 1B and D, Table 1). F4/80+ cell infiltration was significantly increased in the MCD 4w group compared with that in the Control 4w group (Figure 1C and E). In addition, we found that the mice in the MCD 4w group exhibited indications of liver fibrosis, including periportal and interstitial collagen deposition (Figure 2A and C). Immunohistochemical analysis further confirmed our result: α-SMA protein expression was significantly up-regulated in the MCD 4w group compared with that in the Control 4w group (Figure 2B and D). Intestinal barrier function was further impaired in the mice fed the MCD diet for 4 wk compared with that in the mice fed the control diet, as revealed by ZO-1 immunostaining (Figure 3).

MCD diet induced gradual gut microbiota dysbiosis

The MCD diet clearly altered the gut microbiota configuration. No significant differences were observed in alpha diversity between the control and MCD groups after 2 and 4 wk of treatment, as estimated by the Chao1, Shannon, and Simpson indices (data not shown). However, these two groups were clearly separated into different clusters at 2 wk (ANOSIM, $P < 0.01$, r =

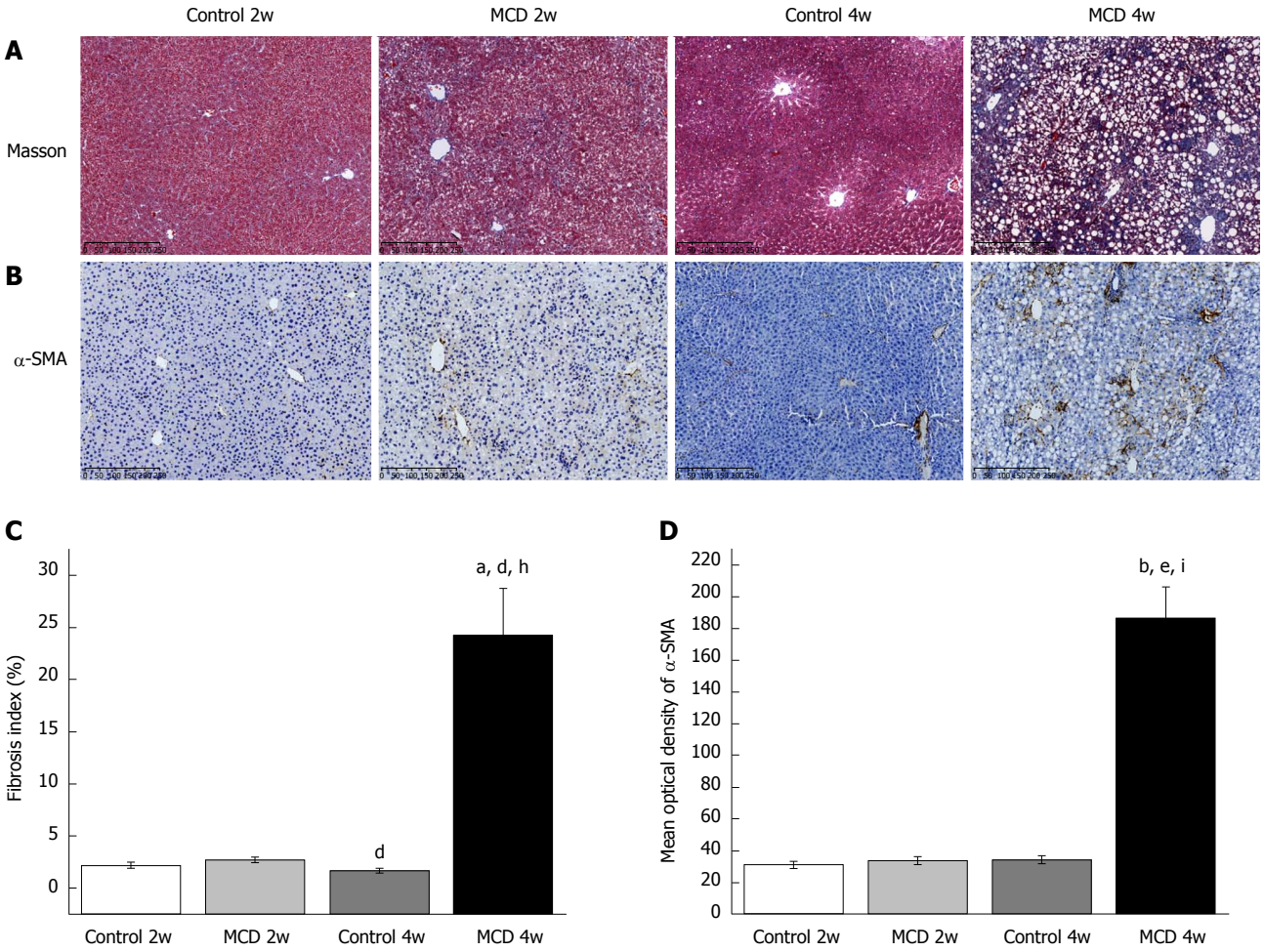


Figure 2 Methionine-choline-deficient diet induced liver fibrosis after 4 wk of feeding. A-B: Representative liver histology assessed by Masson's trichrome staining (A) and α-SMA staining (B). Scale bar: 250 μm. C: Percent of Masson's trichrome-stained area as indicated by the fibrosis index (%). D: Representative staining intensities of α-SMA as designated by the mean optical density. The data are given as the means ± SEM. $n = 6$ per group. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* Control 2w group, $^{d}P < 0.05$ and $^{e}P < 0.01$ *vs* MCD 2w group, $^{h}P < 0.05$ and $^{i}P < 0.01$ *vs* Control 4w group by post hoc ANOVA one-way statistical analysis. MCD: Methionine-choline-deficient.

0.6352) and at 4 wk (ANOSIM, $P < 0.01$, r = 0.9074) in the unweighted UniFrac principal coordinate analysis (PCoA), which was performed to calculate the beta-diversity values (Figure 4A). The most abundant taxa at the phylum, family, and genus levels are shown in Figure 4B-D. At the phylum level, Tenericutes was increasingly more abundant in the fecal microbiota of the MCD group compared with that of the control group at 2 and 4 wk ($P < 0.05$, and $P < 0.01$, respectively), while Verrucomicrobia was consistently less abundant in the MCD group at 2 and 4 wk ($P < 0.05$, and $P < 0.05$, respectively). Compared with the control group, the MCD group had a significantly higher abundance of Firmicutes and a significantly reduced abundance of Proteobacteria at 2 wk ($P < 0.001$, and $P < 0.05$, respectively). At 4 wk, the abundance of Actinobacteria was significantly lower in the MCD group than that in the corresponding control group ($P < 0.01$). At the family level, the relative abundance levels of *Rikenellaceae*, *Desulfovibrionaceae*, and *Verrucomicrobiaceae* were persistently reduced in the MCD group compared with those in the control group at 2 wk ($P < 0.001$, $P < 0.05$, and $P < 0.05$ respectively) and at 4 wk ($P < 0.05$, $P < 0.05$, and $P < 0.05$ respectively). The relative abundance of the Bacteroidales S24-7 group was significantly lower in the MCD group than that in the control group at 2 wk ($P < 0.01$), while *Ruminococcaceae* was significantly higher in the MCD group ($P < 0.05$). Compared with the control group, the MCD group had significantly higher abundance levels of *Bacteroidaceae* and *Enterobacteriaceae* at 4 wk ($P < 0.01$, and $P < 0.05$ respectively) and a significantly reduced abundance of *Bifidobacteriaceae* ($P < 0.05$). At the genus level, the abundance of the Rikenellaceae RC9 gut group was significantly reduced in the MCD group compared with that in the control group at 2 and 4 wk ($P < 0.001$, and $P < 0.05$, respectively). Compared with the control group, the MCD group presented with marked decreases in the abundance levels of *Alistipes* and (Eubacterium) coprostanoligenes at 2 wk ($P < 0.001$, and $P < 0.05$, respectively) and a significant increase in Ruminococcaceae UCG 014 abundance ($P < 0.05$). However, at 4 wk, the MCD diet significantly reduced the

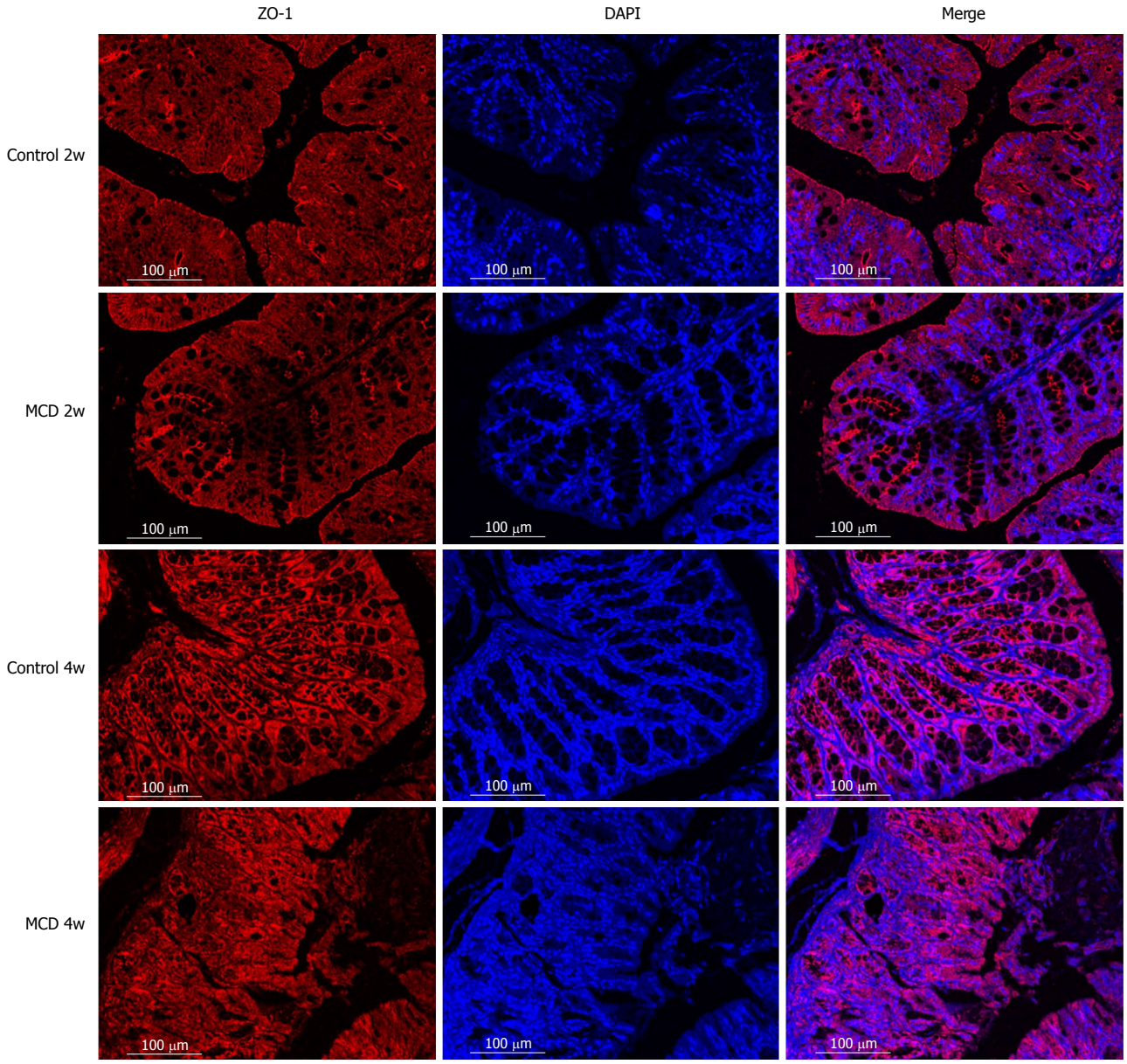


Figure 3 Methionine-choline-deficient diet resulted in gradual intestinal barrier impairment. Representative colon histology was assessed by ZO-1 immunofluorescence staining. Scale bar: 100 μm. The data are given as the means ± SEM. MCD: Methionine-choline-deficient.

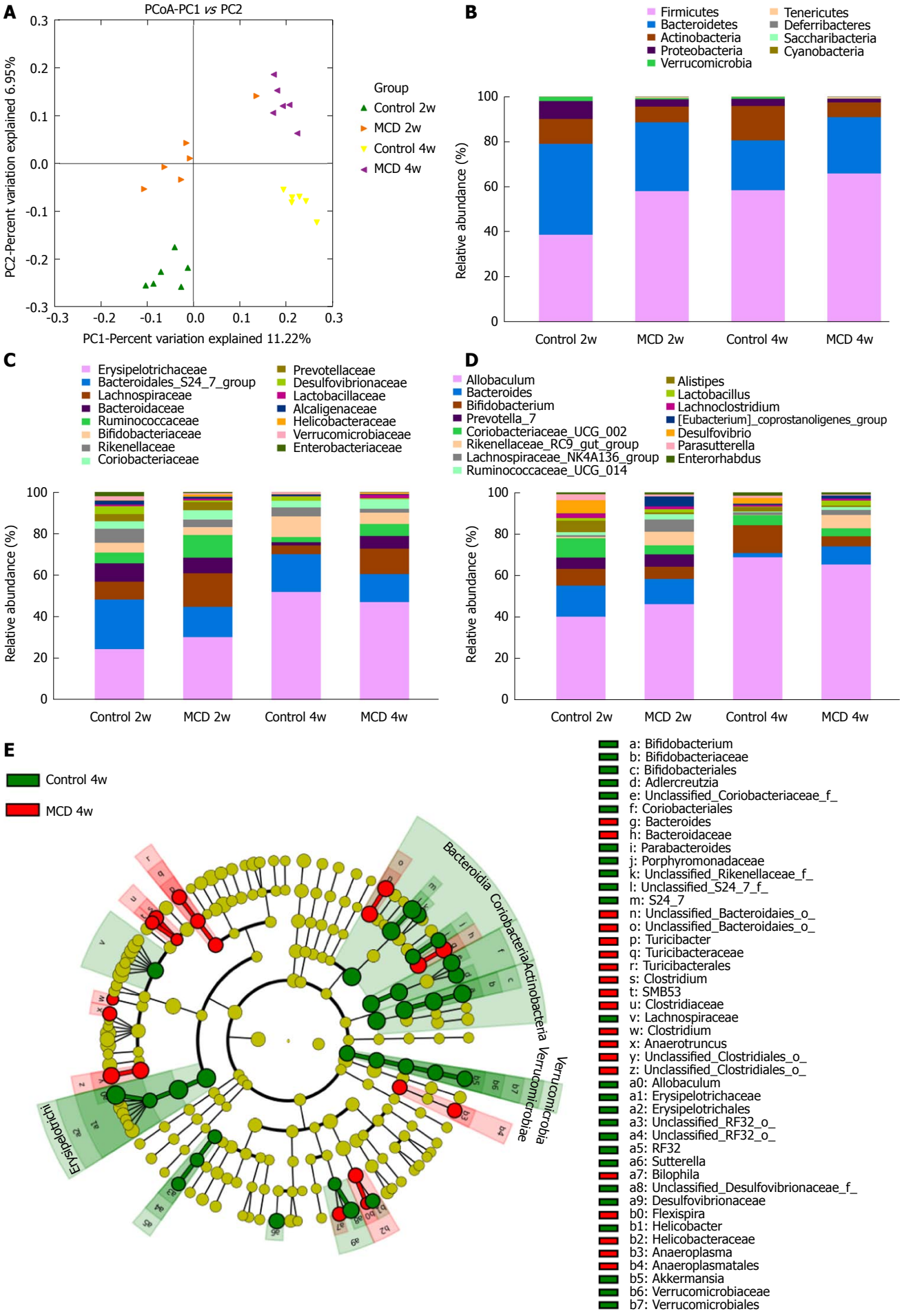
abundance of the promising probiotic *Bifidobacterium* and markedly promoted *Bacteroides* abundance ($P < 0.05$, and $P < 0.01$, respectively).

To characterize further the distinguishing phylotypes in the gut microbiota of the two groups, LEfSe analysis was performed. No significant differences were found between the MCD 2w group and Control 2w group. At 4 wk, however, we found that the MCD diet increased the abundance levels of *Anaerotruncus*, *Bilophila*, *SMB53*, *Clostridium*, *Anaeroplasma*, *Turicibacter*, *Helicobacteraceae*, *Flexispira*, and *Bacteroides* [LDA score (-log10) > 4.8] and decreased the abundance levels of *Allobaculum*, S24-7, *Bifidobacterium*, *Adlercreutzia*, *Lachnospiraceae*, *Akkermansia*, *Sutterella*, *Desulfovibrionaceae*, *Porphyromonadaceae*, *Parabacteroides*, and *Erysipelotrichaceae* [LDA score ($\log_{10}$) > 4.8] (Figure 4E and F).

MCD diet altered the fecal metabolomic profile during NASH progression

Using an untargeted strategy, we studied the fecal metabolome associated with functional characteristics of the gut microbiome, and a total of 322 metabolites were ultimately identified and quantified.

The PCA model was established ($R^2X = 0.526$, $Q^2 = 0.223$), corresponding to the four groups (the Control 2w group, MCD 2w group, Control 4w group, and MCD 4w group) (Figure 5A), and the score plot showed a clustering tendency in the first predictive principal component (X axis) and second predictive principal component (Y axis). Then, an OPLS-DA model was constructed. As depicted in Figure 5B, the score plot in the direction of the X axis (the first predictive principal component) and Y axis (the first orthogonal principal


A
PCoA-PC1 vs PC2
PC2-Percent variation explained 6.95%
PC1-Percent variation explained 11.22%
Group
Control 2w
MCD 2w
Control 4w
MCD 4w
B
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Verrucomicrobia
Tenericutes
Deferribacteres
Saccharibacteria
Cyanobacteria
Relative abundance (%)
Control 2w
MCD 2w
Control 4w
MCD 4w
C
Erysipelotrichaceae
Bacteroidales_S24_7_group
Lachnospiraceae
Bacteroidaceae
Ruminococcaceae
Bifidobacteriaceae
Rikenellaceae
Coriobacteriaceae
Prevotellaceae
Desulfovibrionaceae
Lactobacillaceae
Alcaligenaceae
Helicobacteraceae
Verrucomicrobiaceae
Enterobacteriaceae
Relative abundance (%)
Control 2w
MCD 2w
Control 4w
MCD 4w
D
Allobaculum
Bacteroides
Bifidobacterium
Prevotella_7
Coriobacteriaceae_UCG_002
Rikenellaceae_RC9_gut_group
Lachnospiraceae_NK4A136_group
Ruminococcaceae_UCG_014
Alistipes
Lactobacillus
Lachnoclostridium
[Eubacterium]_coprostanoligenes_group
Desulfovibrio
Parasutterella
Enterorhabdus
Relative abundance (%)
Control 2w
MCD 2w
Control 4w
MCD 4w
E
Control 4w
MCD 4w
Bacteroidia
Coriobacteriia
Actinobacteria
Verrucomicrobiae
Verrucomicrobia
Erysipelotrichi
a: Bifidobacterium
b: Bifidobacteriaceae
c: Bifidobacteriales
d: Adlercreutzia
e: Unclassified_Coriobacteriaceae_f_
f: Coriobacteriales
g: Bacteroides
h: Bacteroidaceae
i: Parabacteroides
j: Porphyromonadaceae
k: Unclassified_Rikenellaceae_f_
l: Unclassified_S24_7_f_
m: S24_7
n: Unclassified_Bacteroidaies_o_
o: Unclassified_Bacteroidaies_o_
p: Turicibacter
q: Turicibacteraceae
r: Turicibacterales
s: Clostridium
t: SMB53
u: Clostridiaceae
v: Lachnospiraceae
w: Clostridium
x: Anaerotruncus
y: Unclassified_Clostridiales_o_
z: Unclassified_Clostridiales_o_
a0: Allobaculum
a1: Erysipelotrichaceae
a2: Erysipelotrichales
a3: Unclassified_RF32_o_
a4: Unclassified_RF32_o_
a5: RF32
a6: Sutterella
a7: Bilophila
a8: Unclassified_Desulfovibrionaceae_f_
a9: Desulfovibrionaceae
b0: Flexispira
b1: Helicobacter
b2: Helicobacteraceae
b3: Anaeroplasma
b4: Anaeroplasmatales
b5: Akkermansia
b6: Verrucomicrobiaceae
b7: Verrucomicrobiales

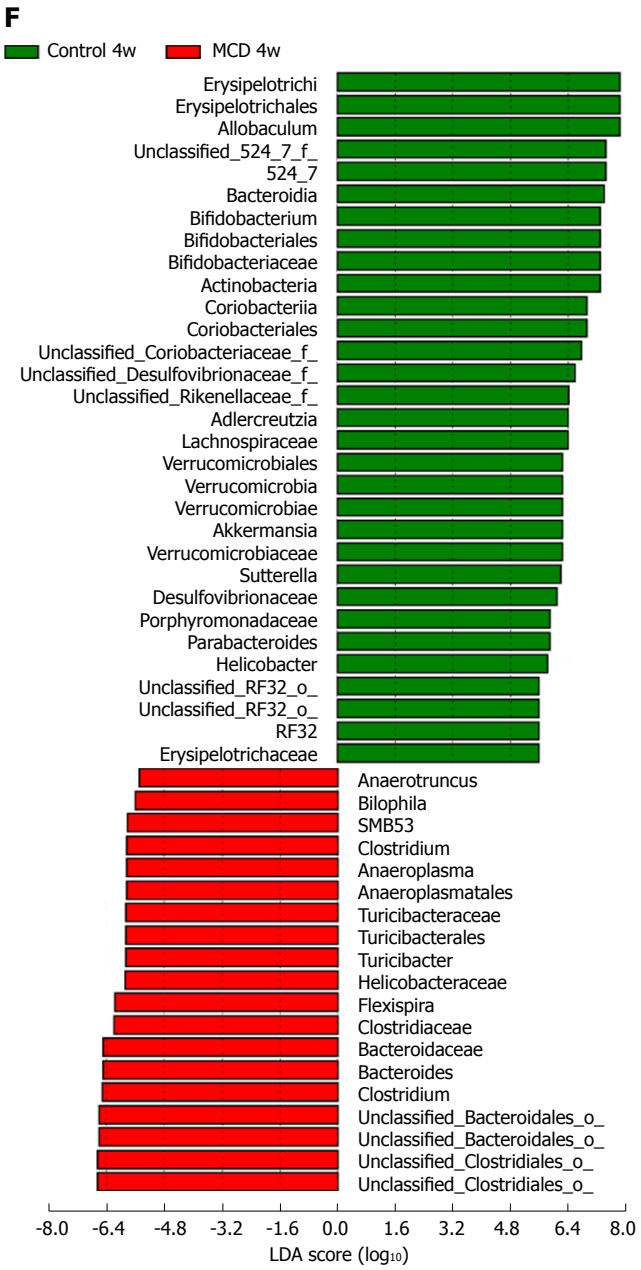


Figure 4 Methionine-choline-deficient diet induced gut microbiota dysbiosis. A: PCoA plot of the microbiota based on unweighted UniFrac metric. Each symbol represents one sample (n = 6 per group). B-D: Top most abundant taxa at the phylum (B), family (C), and genus (D) levels. E: LEfSe cladogram represented taxon enriched in the Control 4w group (green) and in the MCD 4w group (red). Rings from the inside out represented taxonomic levels from phylum to genus. Sizes of the circles indicate the relative abundance levels of the taxa. F: Discriminative biomarkers with an LDA score > 4.8. MCD: Methionine-choline-deficient.

component) showed a significant separation in the metabolomics datasets among the four groups. The explained variance, R^2, was 0.968. The cross-validated predictive ability Q^2 was 0.914, indicating that a random fecal GC-MS spectrum discriminates among the four groups 91.4% of the time. Therefore, these results indicate the distinct clustering of the fecal metabolomic profiles induced by the MCD diet over time. Characteristic metabolites that had been significantly modified by the MCD diet were further identified according to the OPLS-DA model, with VIP values > 1 and P values < 0.05 (Figure 5E and F, and Supplementary Tables 2 and 3). Ultimately, 103 and 93 metabolites were selected at 2 and 4 wk, respectively, most of which were mainly associated with pathways involved in lipid, amino acid, carbohydrate, nucleotide, cofactors, and vitamin metabolism. S-plots were used to identify further potential biomarkers among the metabolites. As shown in Figure 5C and D, arachidic acid, hexadecane, palmitic acid, and tetracosane at 2 wk and cholic acid, cholesterol, arachidic acid, tetracosane, and stearic acid at 4 wk were the farthest from the origin and were selected as potential biomarkers due to their marked contribution to the separation between the control and MCD groups.

DISCUSSION

To the best of our knowledge, this is the first study to

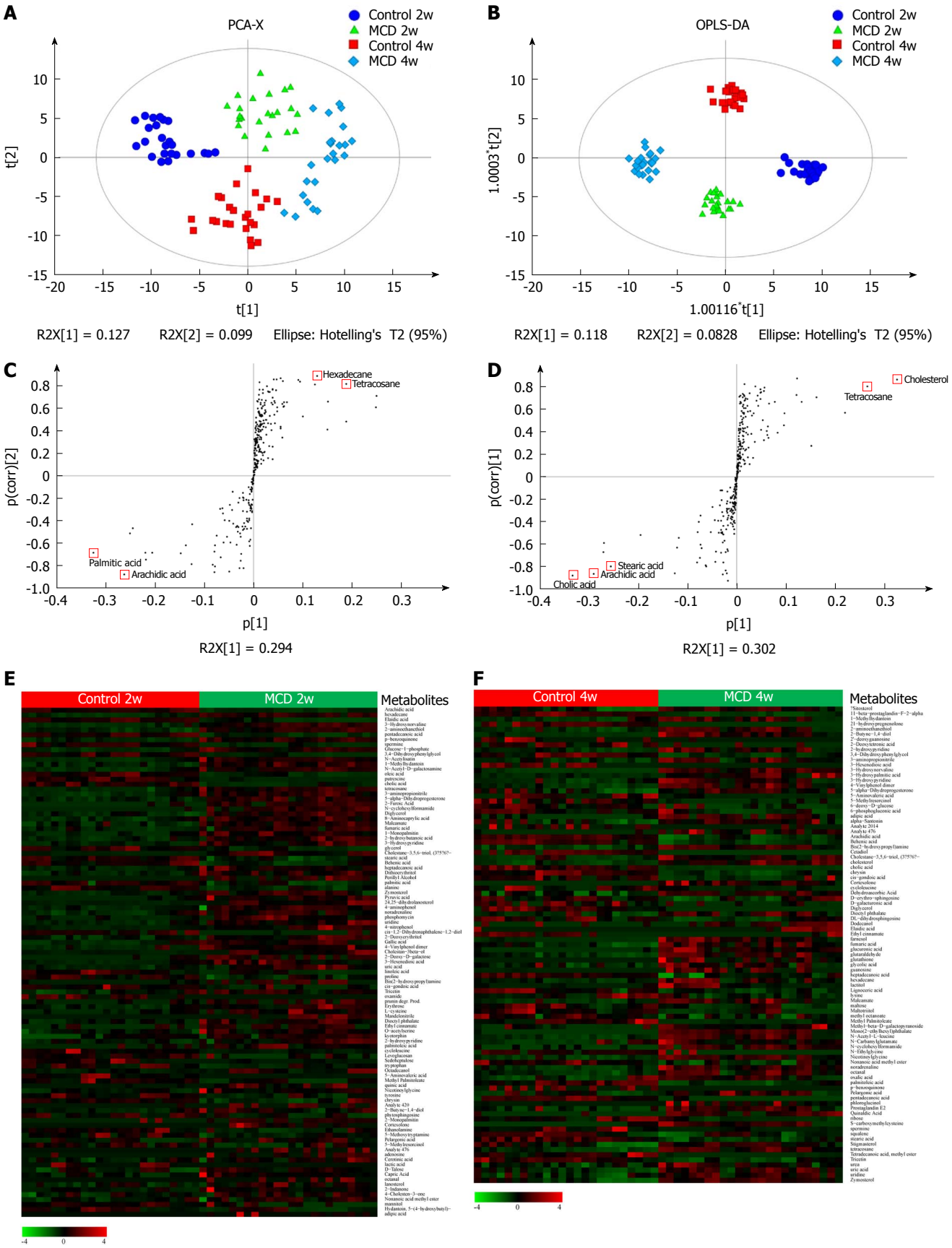


Figure 5 Methionine-choline-deficient diet altered the fecal metabolomic profile during nonalcoholic steatohepatitis progression. A: 4-state model of a PCA plot comparing between the Control 2w (cycle), MCD 2w (triangle), Control 4w (square), and MCD 4w groups (diamond). B: OPLS-DA score scatter plot comparing the Control 2w (cycle), MCD 2w (triangle), Control 4w (square), and MCD 4w groups (diamond). Each symbol represents one sample (n = 24 per group, with a quadruplicate technical replicates of 6 samples per group). C-D: S-plot constructed from the OPLS-DA model at 2 wk (C) and 4 wk (D). The selected metabolites (red triangles) are located in the upper far right and lower far left with considerable potential as biomarkers and marked discrimination between the groups. E-F: Euclidean distance hierarchical clustering analysis visualizing the different intensity levels of characteristic metabolites at 2 wk (E) and 4 wk (F). PCA: Principal component analysis; MCD: Methionine-choline-deficient; OPLS-DA: Orthogonal partial least-squares-discriminant analysis.

demonstrate dynamic alterations in the gut microbiota and metabolome in an experimental model of MCD diet-induced steatohepatitis: We sought to determine the key microbiota and metabolites involved in NASH progression over time.

Mice fed the MCD diet developed simple hepatic steatosis at 2 wk and prominent NASH at 4 wk. Among the most abundant taxa, the prevalence of the Rikenellaceae RC9 gut group in the family *Rikenellaceae*, family *Desulfovibrionaceae*, and family *Verrucomicrobiaceae* in the phylum Verrucomicrobia was consistently decreased at 2 and 4 wk in the MCD-supplemented group compared with that in the control group, while the phylum Tenericutes was consistently increased in the MCD-supplemented group. The phylum Tenericutes was also increased in the mice fed a high-fat diet and is correlated with obesity-associated metabolic characteristics[21]. The Rikenellaceae RC9 gut group was previously shown to be decreased in rats with hypertriglyceridemia-related acute necrotizing pancreatitis and in cows receiving oil supplementation[22,23], but the specific function of this taxa remains poorly understood. High *Rikenellaceae* abundance is reportedly associated with healthy metabolic states[24], and one study reported the ability of some of the bacteria in the family *Rikenellaceae* to produce butyrate[25]. *Desulfovibrionaceae* are known as sulfate-reducing bacteria that produce hydrogen sulfide (H_2S)[26], which has its pros and cons in relation to gut health. Some studies have demonstrated that H_2S functions as a mucosal barrier breaker and therefore leads to inflammation[27,28]. Regarding the advantages of H_2S, previous studies have reported that H_2S is a crucial mediator in gastrointestinal mucosal defense and repair and in reducing systemic inflammation[26,29]. *Verrucomicrobiaceae* abundance is positively correlated with gastrointestinal health and negatively correlated with gut inflammation[30]. Therefore, disrupted barrier homeostasis, as evidenced by the ZO-1 immunostaining results in this study, may be associated with a decrease in MCD-sensitive microbiota.

In this study, we found that *Bifidobacterium* in the family *Bifidobacteriaceae*, *Bacteroides* in the family *Bacteroidaceae*, and the family *Enterobacteriaceae* respond to the MCD diet in a time-dependent manner. The first of these taxa tended to decrease, while the latter two tended to increase after only 4 wk of feeding. Consistently, *Bifidobacterium* was significantly decreased in NASH subjects compared with that in healthy and obese subjects[10]. Various members of the genus *Bifidobacterium* have recently attracted substantial interest due to their multiple beneficial effects on the host, such as protection against enteropathogenic infection through acetate production[31], promotion of antitumor immunity, and facilitation of programmed cell death protein 1 ligand 1 (PD-L1)-specific antibody therapy[32]. Although *Bacteroides* spp. are overall regarded as beneficial microorganisms[33], they can also cause gut-related bacteremia in conditions of high portal venous pressure[34]. *Enterobacteriaceae* include many potential pathogens and LPS-producing bacteria and are significantly increased in NASH patients[10]. Furthermore, as evidenced by LEfSe analysis, the MCD diet favored LPS-producing bacteria (*Bilophila*, and *Anaeroplasma*[35,36]), pro-inflammatory bacteria (*Anaerotruncus*, and *Turicibacter*[37,38]), and other opportunistic pathogens (*SMB53*, *Clostridium*, *Helicobacteraceae* and *Flexispira*[39-42]) after 4 wk of feeding and inhibited the promising probiotics *Sutterella* and *Akkermansia*[43,44]. Therefore, these data indicate an important role for the microbiota in NAFLD progression to NASH.

To link the gut microbiota with their functional states, an untargeted metabolomics analysis was integrated into this study. A core of the MCD diet-responsive signaling pathways were involved in lipid, nucleotide, cofactor, vitamin, carbohydrate, and amino acid metabolism. S-plots further identified potential biomarkers among the metabolites during NASH development. Arachidic acid under-representation and tetracosane over-representation were prominent features in the group with MCD diet-induced steatosis and NASH compared with the corresponding control groups at 2 and 4 wk. However, functional exploration of the association between arachidic acid and tetracosane with non-alcoholic fatty liver disease has not been well documented and warrants further studies. Kuroda *et al*[45] reported that hexadecane causes nonspecific inflammation. Therefore, we speculated in this study that the increased hexadecane levels after the MCD diet treatment for 2 wk may contribute to disease progression, although we have not yet obtained direct evidence for this effect. Jiao *et al*[46] found that the cholic acid level is increased in NASH patients. JianHua *et al*[47] found that cholic acid is significantly decreased in humans with NASH, which is consistent with our study results in the MCD 4w group. Stearic acid is a potent anti-inflammatory lipid and may accelerate hepatic dysfunction recovery in a rat model of liver injury[48]. Our study found that the stearic acid level was significantly decreased in the NASH mice after the MCD diet treatment for 4 wk. Although our study found an association between altered gut microbiota and metabolism and NASH, a causative contribution of the gut microbiota and metabolism to NASH progression has not been sufficiently documented. Establishing a better understanding of the gut microbiota and metabolomics in this disease state will provide beneficial information for the treatment and prevention of NAFLD.

In conclusion, the MCD diet induced gut microbiota and metabolome deterioration. Fundamental observations of these alterations will provide new insight into NASH-associated intestinal disorder and gut-targeted therapies for NASH.

ARTICLE HIGHLIGHTS

Research background

The contributing role of the gut microbiota in the pathogenesis of nonalcoholic

steatohepatitis (NASH) has been extensively studied.

Research motivation

Gut microbiota dysbiosis in NASH is mainly depicted as an endpoint, and little is known regarding the microbiota disturbances during NASH progression.

Research objectives

Our goal was to investigate dynamic changes in the gut microbiota and its metabolism during the progression from simple hepatic steatosis to NASH in mice fed a methionine-choline-deficient (MCD) diet.

Research methods

C57BL/6J mice were equally divided into four groups and fed either a methionine-choline-sufficient diet for 2 or 4 wk (the Control 2w group and Control 4w group, respectively) or the MCD diet for 2 or 4 wk (the MCD 2w group and MCD 4w group, respectively) (n = 6 per group). Liver injury, fibrosis, intestinal barrier function, and the fecal microbiome and metabolome were studied.

Research results

The mice fed with the MCD diet for 2 wk developed simple hepatic steatosis, which progressed to prominent NASH with liver fibrosis after 4 wk. Compared with the control diet, the MCD diet induced gradual intestinal barrier impairment and gut microbiota dysbiosis; the fecal metabolomic profile was also substantially altered by the MCD diet.

Research conclusions

The MCD diet induced persistent alteration of the gut microbiota and metabolome.

Research perspectives

We may have for the first time shown that an MCD diet induced persistent gut microbiota and metabolome deterioration. Fundamental observations of these alterations will provide new insight into NASH-associated intestinal disorder and gut-targeted therapies for NASH.

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ORIGINAL ARTICLE

Case Control Study

Association of twelve polymorphisms in three onco-lncRNA genes with hepatocellular cancer risk and prognosis: A case-control study

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Abstract

AIM

To evaluate the association of 12 tag single nucleotide polymorphisms (tagSNPs) in three onco-long non-coding RNA (lncRNA) genes (*HOTTIP*, *CCAT2*, *MALAT1*) with the risk and prognosis of hepatocellular cancer (HCC).

METHODS

Twelve tagSNPs covering the three onco-lncRNAs were genotyped by the KASP method in a total of 1338 samples, including 521 HCC patients and frequency-matched 817 controls. The samples were obtained from an unrelated Chinese population at the First Hospital of

China Medical University from 2012-2015. The expression quantitative trait loci (eQTL) analyses were conducted to explore further the potential function of the promising SNPs.

RESULTS

Three SNPs in *HOTTIP*, one promoter SNP in *MALAT1*, and one haplotype of *HOTTIP* were associated with HCC risk. The *HOTTIP* rs17501292, rs2067087, and rs17427960 SNPs were increased to 1.55-, 1.20-, and 1.18-fold HCC risk under allelic models (P = 0.012, 0.017 and 0.049, respectively). *MALAT1* rs4102217 SNP was increased to a 1.32-fold HCC risk under dominant models (P = 0.028). In addition, the two-way interaction of *HOTTIP* rs17501292-*MALAT1* rs619586 polymorphisms showed a decreased effect on HCC risk ($P_{interaction}$ = 0.028, OR = 0.30) and epistasis with each other. *HOTTIP* rs3807598 variant genotype showed significantly longer survival time in HBV negative subgroup (P = 0.049, HR = 0.12), and *MALAT1* rs591291 showed significantly better prognosis in female and HBV negative subgroups (P = 0.022, HR = 0.37; P = 0.042, HR = 0.25, respectively). In the study, no significant effect was observed in eQTL analysis.

CONCLUSION

Specific lncRNA (*HOTTIP* and *MALAT1*) SNPs have potential to be biomarkers for HCC risk and prognosis.

Key words: Hepatocellular cancer; Single nucleotide polymorphism; Long non-coding RNA; Risk; Prognosis

Core tip: We aim to evaluate the association of twelve tag single nucleotide polymorphisms (tagSNPs) in three onco-lncRNA genes (*HOTTIP*, *CCAT2*, *MALAT1*) with the risk and prognosis of hepatocellular cancer (HCC). Twelve tagSNPs covering the three onco-lncRNAs were genotyped by the KASP method in a total of 1338 samples. We found three SNPs in *HOTTIP*, one promoter SNP in *MALAT1* and one haplotype of *HOTTIP* gene were associated with HCC risk. In addition, *HOTTIP* rs3807598 variant genotype showed significantly longer survival time in hepatitis B virus (HBV) negative subgroup, and *MALAT1* rs591291 showed significantly better prognosis in female and HBV negative subgroups.

Wang BG, Xu Q, Lv Z, Fang XX, Ding HX, Wen J, Yuan Y. Association of twelve polymorphisms in three onco-lncRNA genes with hepatocellular cancer risk and prognosis: A case-control study. *World J Gastroenterol* 2018; 24(23): 2482-2490 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2482.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2482

INTRODUCTION

Hepatocellular cancer (HCC) is a common malignant tumor with high incidence and mortality, and it is the main histologic type of primary liver cancer[1,2]. Similar to most other solid tumors, HCC patients are considered to be incurable due to the extensive heterogeneity in the clinical manifestations and biological characteristics[3]. HCC heterogeneity can be manifested via diverse genetic, epigenetic, and histogenic features, as well as ethnic differences in patients[4]. Knowledge of genetic and epigenetic variations could aid the early detection and personalized management of HCC. To date, the research hotspots regarding genetic and epigenetic variations are not only coding genes but also noncoding RNAs.

Long non-coding RNAs (lncRNAs) are a type of noncoding RNA with a length of 200 bp, which can function as miRNA sponges to compete with mRNAs by acting as so-called competing endogenous RNAs (ceRNAs)[5]. Genetic variations such as single-nucleotide polymorphisms (SNPs) can alter the expression of coding genes and lncRNAs[6]. To date, several lncRNAs have been reported to be involved in carcinogenesis, such as *H19*, *HOTHAIR*, *HOTTIP*, *CCAT2*, and *MALAT1*. Regarding studies of genetic variation, only *H19* and *HOTAIR* SNPs have been well investigated. For example, several meta-analyses showed that *H19* and *HOTHAIR* SNPs were associated with cancer risk[7-9], and the *HOTAIR* rs920778 SNP was found to be associated with ovarian cancer prognosis[10]. Only four studies have focused on the polymorphisms of the onco-lncRNAs *HOTTIP*, *CCAT2*, and *MALAT1* (Gene ID: 100316868, 101805488, 378938, respectively)[11-14]. Among them, Gong *et al*[11] found that these SNPs were significantly associated with lung cancer susceptibility or platinum-based chemotherapy response. However, no SNPs in the above-mentioned lncRNAs have been reported to be associated with HCC risk, and few comprehensive and systematic analyses have been performed on polymorphisms in these three onco-lncRNAs. It thus remains unclear whether the promising SNPs in these lncRNAs have potential to be used as biomarkers for HCC risk and prognosis.

In the present study, we adopted a candidate gene association study strategy with the selected 12 potentially functional tagSNPs covering the three onco-lncRNAs *HOTTIP*, *CCAT2*, and *MALAT1* to determine whether these SNPs are associated with HCC risk and prognosis and whether promising SNPs could affect the expression of corresponding lncRNAs. We aimed to identify predictive biomarkers for HCC risk and prognosis, establish experimental basis for the comprehension of the HCC etiology, and improve our understanding of the pathogenesis and disease progression of HCC.

MATERIALS AND METHODS

Patients and study design

This research project was approved by the Ethical Committee of the First Hospital of China Medical University and written informed consent was obtained from each subject at the time of recruitment. The study

was designed to be composed of two parts: risk and prognosis. In the risk study, a total of 1338 participants were recruited, including 521 patients who underwent surgical operation for HCC at the First Hospital of China Medical University between 2012 and 2015. The inclusion and exclusion criteria were as follows: (1) The participants who underwent surgical operation were diagnosed with HCC by pathological confirmation, in accordance with the World Health Organization (WHO) classification; and (2) removal of other pathological types of liver cancer (gallbladder cell carcinoma, mix-type liver cancer. and hepatosarcoma). A total of 817 frequency-matched controls were also recruited, some of whom were from a health screening program from the Zhuanghe area, Liaoning Province, China, performed between 2002 and 2012, while others were from a health screening program at the First Hospital of China Medical University performed between 2012 and 2015.

To investigate further the association of these lncRNA polymorphisms with clinicopathological parameters and overall survival of HCC patients, we used data of 351 HCC cases for which information on death or survival was available. Patients with (1) distant metastasis found preoperatively or (2) incomplete pathological data entries were excluded from the survival analysis. Follow-up was completed by July 1st, 2017. For the promising SNPs, lncRNA expression was investigated to explore the possible mechanism by which they exerted their effects, according to our experimental and bioinformatic data. The study design is shown in Figure 1. This research project was approved by the Ethical Committee of the First Hospital of China Medical University and written informed consent was obtained when the patients and controls were recruited.

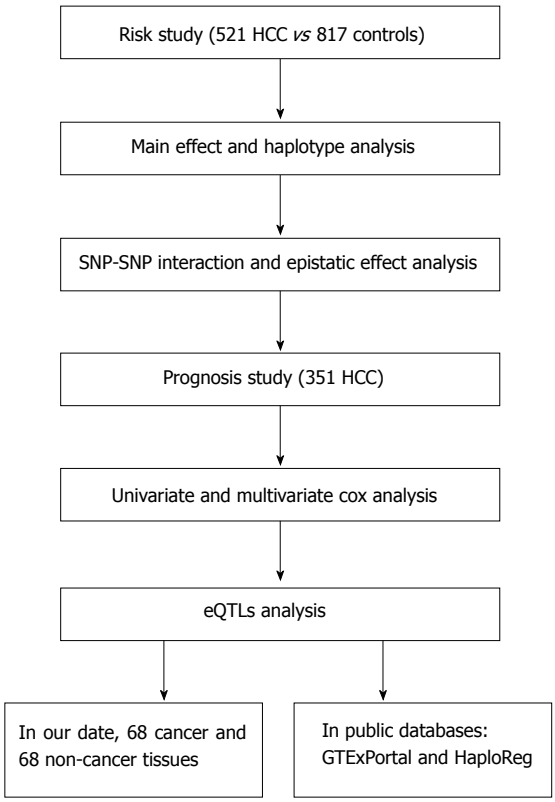

Figure 1 Flow chart of the study design.

Selected polymorphic sites

We selected polymorphisms using 1000G data (http://www.internationalgenome.org/home), as reported previously[15-17]. The tagSNPs were selected separately using the following criteria: (1) Haploview with the Tagger function was used; (2) the population of the HapMap selected Chinese Han Beijing (CHB) population; (3) those for which pairwise tagging had r^2 of ≥ 0.8; and (4) those with a minor allele frequency of ≥ 5%. The selection area was enlarged by 10 kb both upstream and downstream for these three lncRNA genes. FastSNP and fSNP searches were used to predict the potential SNP function (http://compbio.cs.queensu.ca/F-SNP/)[18,19]. A total of 12 SNPs from the three lncRNA genes were selected by integrating these two publicly available tools. Locations and characteristics of the selected SNPs are shown in Table 1.

Genotyping

Genomic DNA was extracted using a previously reported method[20] and diluted to a working concentration of 20 ng/μL for genotyping. The genotyping assay was performed by Gene Company (Shanghai, China), using allele-specific PCR with KASPar (KASP) reagents (LGC Genomics, Hoddesdon, United Kingdom). For quality control, we repeatedly genotyped 10% of the total samples at one time. The concordance rate of these repeated samples reached 100%, which demonstrated that the genotyping results were reliable.

eQTL analyses

The extraction of total RNA from 68 HCC specimens and corresponding samples from nearby noncancerous regions was performed as described previously[15], and a total of 2.0 μg of isolated RNA was converted into cDNA using Quantscript RT Kit (Tiangen Biotech, Beijing, China). The RNA expression levels of the promising lncRNA genes (*HOTTIP* and *MALAT1*) and an internal-control gene (GAPDH) were measured using SYBR Premix Ex Taq II (TaKaRa Biotech, Dalian, China) in an Eppendorf Mastercycler Gradient System (Eppendorf AG, Hamburg, Germany). Each reaction was performed in duplicate and controls without a template were also tested every time. The primers are summarized in Supplementary Table 1.

To perform functional candidate polymorphism and expression quantitative trait locus (eQTL) analyses on the promising genes, we mined the data from the following databases: GTExPortal (https://www.gtexportal.org/home/) and Haploreg (http://www.broadinstitute.org/mammals/haploreg/haploreg.php)[21].

Statistical analysis

Between-group differences in sex variability, as well as accordance with Hardy-Weinberg equilibrium, were

Table 1 Association of lncRNA gene single nucleotide polymorphisms and risk of hepatocellular cancer (*n*)

Gene	Chr. Pos.	SNP[1]	Loc.	Genotype	Controls (%)	Cases (%)	*P*[2] value	OR (95%CI)	P_{HWE} value
HOTTIP	7p15.2	rs17501292	Exon	TT	732 (91.2)	453 (87.1)		1 (Ref.)	0.190
				TG	71 (8.8)	66 (12.7)	0.021	1.52 (1.06-2.17)	
				GG	0 (0)	1 (0.2)	NA	NA	
				TG + GG *vs* TT			0.017	1.54 (1.08-2.20)	
				G *vs* T			0.012	1.55 (1.10-2.18)	
		rs2067087	Exon	GG	174 (21.7)	88 (16.9)		1 (Ref.)	0.674
				GC	405 (50.6)	263 (50.7)	0.236	1.16 (0.90-1.49)	
				CC	222 (27.7)	168 (32.4)	0.015	1.49 (1.08-2.08)	
				CC *vs* GG + GC			0.035	1.35 (1.02-1.82)	
				C *vs* G			0.017	1.20 (1.03-1.41)	
		rs17427960	Intron	CC	172 (21.7)	85 (16.7)		1 (Ref.)	0.613
				CA	387 (48.8)	259 (50.9)	0.707	1.05 (0.81-1.35)	
				AA	234 (29.5)	165 (32.4)	0.032	1.78 (1.03-2.00)	
				AA *vs* CC + CA			0.028	1.39 (1.03-1.85)	
				A *vs* C			0.049	1.18 (1.00-1.37)	
MALAT1	22q13.2	rs4102217	Promoter	GG	608 (75.1)	362 (69.6)		1 (Ref.)	0.055
				GC	180 (22.2)	148 (28.5)	0.011	1.39 (1.08-1.79)	
				CC	22 (2.7)	10 (1.9)	0.481	0.76 (0.36-1.63)	
				GC + CC *vs* GG			0.028	1.32 (1.03-1.69)	
				C *vs* G			0.097	1.20 (0.97-1.50)	

[1]The sort order was according to the SNP location in its genes from 5' starting to 3' ends. [2]*P* value was calculated by adjusted by age and gender. NA: Not available; Chr. Pos.: Chromosomal position; Loc.: Localisation; P_{HWE}: *P* value for Hardy-Weinberg Equilibrium.

compared by the χ^2 test and by analysis of variance for age variability. Multivariate logistic regression with adjustments for age and sex was used to show the association between selected lncRNA polymorphisms and HCC risk. The haplotypes of each gene were analyzed using SHEsis software[22]. The two-way pairwise interactions of lncRNA SNP-SNP were calculated using multivariate logistic regression. Univariate and multivariate survival analyses were carried out by the log-rank test and the Cox proportional hazards model. The differences of relative lncRNA levels between two groups were tested by Student's *t*-test. *P* value < 0.05 was considered to be significant.

RESULTS

The association of SNPs in lncRNA genes with HCC risk

The demographic characteristics of HCC cases and control subjects are shown in Supplementary Table 2. All polymorphism genotype distributions in cases and controls are shown in Table 1, including 12 SNPs in three lncRNA genes (*HOTTIP*: rs3807598, rs17501292, rs2067087, rs17427960, rs78248039; *CCAT2*: rs3843549, rs138947056, rs6983267; *MALAT1*: rs4102217, rs591291, rs11227209, and rs619586). Among them, most SNPs accorded with Hardy-Weinberg equilibrium, except for *CCAT2* rs6983267 (P_{HWE} = 0.029). This SNP was thus excluded from further association analysis.

Among these remaining 11 SNPs, four in the *HOTTIP* and *MALAT1* genes were associated with HCC risk, and they all increased HCC risk (*HOTTIP*: rs17501292, rs2067087, rs17427960; *MALAT1*: rs4102217; Table 1, Supplementary Table 3). Among them, two SNPs (*HOTTIP*: rs17501292; *MALAT1*: rs4102217) showed significant in a dominant model and the other two showed in a recessive model. *HOTTIP* rs17501292 and *MALAT1* rs4102217 were associated with an increased risk of HCC (*P* = 0.017 and 0.028, OR = 1.54 and 1.32, respectively) in a dominant model. In addition, *HOTTIP* rs2067087 and rs17427960 variant genotypes also showed associations with an increased risk of HCC (*P* = 0.035 and 0.028, OR = 1.35 and 1.39, respectively) in a recessive model. Stratified analysis based on gender, age, smoking, and drinking was performed to analyze the association between each SNP and HCC risk. The results are shown in Supplementary Table 4 and suggest these variables have potential predictive value for specific subgroup populations in HCC risk.

The association of haplotype in four lncRNA genes with HCC risk

We chose to exclude haplotypes with a frequency of less than 0.03 from the analysis. We found only one haplotype in the *HOTTIP* gene that was associated with HCC risk. Compared with other haplotypes, patients with the C-G-T-A haplotype of *HOTTIP* rs3807598-rs17501292-rs2067087-rs17427960 showed a 1.91-fold increased risk of HCC (*P* = 0.006, 95%CI: 1.20-3.05; Table 2).

Two-way SNP-SNP interaction models for lncRNA polymorphisms

For the data mining of two-way SNP-SNP interactions, we analyzed all possible pair combinations between all of these 11 SNPs and found that the pairwise interaction of *HOTTIP* rs17501292-*MALAT1* rs619586 was significant ($P_{interaction}$ = 0.028, OR = 0.30, 95%CI: 0.10-0.88; Table 3).

We further analyzed the epistatic effect of *HOTTIP* rs17501292 and *MALAT1* rs619586 and found in the

Table 2 Association of haplotype of lncRNA gene and hepatocellular cancer risk (*n*)

Haplotype	Control (%)	Case (%)	OR (95%CI)	*P* value
HOTTIP[1]				
CGCA	33.54 (2.4)	407.00 (4.5)	1.91 (1.20-3.05)	0.006
CTGC	663.39 (47.3)	389.97 (43.4)	0.85 (0.71-1.01)	0.066
GTCA	604.75 (43.1)	401.77 (44.7)	1.08 (0.91-1.28)	0.406
CCAT2[2]				
AAG	623.60 (39.8)	434.27 (42.7)	1.13 (0.96-1.33)	0.136
AAT	937.40 (59.8)	577.73 (56.9)	0.89 (0.75-1.04)	0.136
MALAT1[3]				
CTCA	217.99 (13.8)	167.00 (16.3)	1.22 (0.08-1.52)	0.080
GCCA	934.81 (59.3)	599.74 (58.6)	0.97 (0.82-1.14)	0.718
GTCA	296.19 (18.8)	1720 (16.6)	0.86 (0.70-1.06)	0.160
GTGG	85.00 (5.4)	58.94 (5.8)	1.07 (0.76-1.51)	0.689

Haplotype for [1]*HOTTIP* rs3807598-rs17501292-rs2067087-rs17427960; [2]*CCAT2* rs3843549-rs138947056-rs6983267; [3]*MALAT1* rs4102217-rs591291-rs11227209-rs619586.

Table 3 The two-way interaction of *HOTTIP* rs17501292-*MALAT1* rs619586 polymorphisms in the risk of hepatocellular cancer

Variables	*MALAT1* rs619586	
	AA	AG + GG
	HCC *vs* CON (*n* = 335 *vs* 572)	
HOTTIP rs17501292		
TT		
Case/control	372/617	78/107
OR (95%CI)	1	1.21(0.88-1.66)
TG + GG		
Case/control	61/55	6/15
OR (95%CI)	1.84 (1.25-2.71)	0.66 (0.26-1.73)
	$P_{interaction}$ = 0.028, OR (95%CI) = 0.30 (0.10-0.88)	

HCC: Hepatocellular cancer; CON: Control.

subset with *MALAT1* rs619586 AA wild type and *HOTTIP* rs17501292 SNP an increased risk of HCC under a dominant model (*P* = 0.002, OR = 1.85); however, in the subset with the *HOTTIP* rs17501292 TG+GG genotype, the *MALAT1* rs619586 SNP decreased the risk of HCC under a dominant model (*P* = 0.050, OR = 0.36; Supplementary Table 5).

The association of lncRNA SNPs with HCC cancer prognosis

We analyzed the association of all 11 SNPs with the overall survival of HCC patients but found no significant association in either univariate or multivariate Cox proportional hazard analysis (Supplementary Table 6). In the stratified analysis, those with the *HOTTIP* rs3807598 variant genotype were shown to have a significantly longer survival time in the HBV-negative subgroup (*P* = 0.049, HR = 0.12, 95%CI = 0.02-0.99), and *MALAT1* rs591291 showed an association with a significantly better prognosis in the female and HBV-negative subgroups (*P* = 0.022, HR = 0.37, 95%CI = 0.16-0.87; *P* = 0.042, HR = 0.25, 95%CI = 0.07-0.95, respectively; Table 4, Supplementary Table 7).

eQTL analysis

We used eQTL analysis to investigate the effect of the SNPs identified to be associated with HCC risk on lncRNA expression. In neither the cancerous group nor the noncancerous group was a significant difference observed for the effect of the positive SNPs on lncRNA expression levels (Table 5). Among them, only the heterozygote genotype of intronic rs17427960 of the *HOTTIP* gene was associated with higher lncRNA-*HOTTIP* expression, with borderline significance (CA *vs* CC: *P* = 0.063; Table 5). Next, we searched public databases for the SNPs for which positive results were obtained in the eQTL analysis (rs17501292, rs2067087, rs17427960, rs4102217). The results from the GTExPortal showed that rs4102217 is a functional SNP in 34 different tissues, such as pancreas and stomach (Supplementary Table 8), and that rs17501292 is a functional SNP in tibial artery tissue; however, no eQTL data for rs2067087 and rs17427960 were found in the public databases. In addition, in Haploreg, it was shown that these SNPs are associated with several kinds of regulatory motifs if the SNP bases altered (Supplementary Figure 1).

DISCUSSION

In this study, we preliminarily screened all of the tagSNPs covering three onco-lncRNAs, *HOTTIP*, *CCAT2*, and *MALAT1*, for associations with HCC risk and prognosis. We identified four promising risk-associated SNPs, one haplotype, and a two-way pairwise interaction combination associated with HCC risk. We also found that patients carrying the *HOTTIP* rs3807598 and *MALAT1* rs591291 variant genotypes had a longer survival time in the HBV-negative subgroup. Further molecular experiments were also conducted to investigate whether the tagSNPs could affect the expression of the corresponding lncRNAs. Our study provides an experimental basis for seeking predictive biomarkers for the risk and prognosis of HCC.

Table 4 Univariate proportional hazard analysis stratified by host characteristics for the association of *lncRNA* polymorphisms and hepatocellular cancer *n* (%)

Gene	SNP	Stratified	Stratified factors	Genotype	HCC	Death	MST (M)	*P* value	Hazard ratio (95%CI)
HOTTIP	rs3807598				*n* = 136				
		HBV	Positive	CC	30 (22.06)	12	90.0		1 (reference)
				CG	73 (53.68)	23	90.1[b]	0.391	0.74 (0.37-1.48)
				GG	33 (24.26)	11	64.1[b]	0.680	0.84 (0.37-1.91)
					n = 31				
			Negative	CC	10 (32.26)	7	21.0		1 (reference)
				CG	13 (41.94)	4	41.8[b]	0.374	0.60 (0.16-1.97)
				GG	8 (25.80)	1	70.3[b]	0.049	0.12 (0.02-0.99)
					n = 285				
MALAT1	rs591291				*n* = 286				
		Gender	Male	CC	98 (34.27)	39	48.0		1 (reference)
				TC	146 (51.05)	56	56.0	0.865	0.97 (0.64-1.45)
				TT	42 (14.68)	19	47.0	0.678	1.12 (0.65-1.94)
					n = 64				
			Female	CC	22 (34.38)	13	32.0		1 (reference)
				TC	37 (57.81)	9	56.0	0.022	0.37 (0.16-0.87)
				TT	5 (7.81)	0	NA	0.286	0.04 (0.00-16.09)
					n = 139				
		HBV	Positive	CC	54 (38.85)	18	69.0		1 (reference)
				TC	69 (49.64)	21	92.1[b]	0.816	0.93 (0.49-1.74)
				TT	16 (11.51)	7	90.0	0.965	0.98 (0.41-2.35)
					n = 31				
			Negative	CC	8 (25.81)	5	6.0		1 (reference)
				TC	16 (51.61)	4	27.0	0.042	0.25 (0.07-0.95)
				TT	7 (22.58)	3	21.0	0.215	0.40 (0.09-1.71)

HR: Hazard rate; MST: Median survival time (months); NA: Not available.

Table 5 Differences of lncRNA gene mRNA levels in different genotypes in hepatocellular cancer and non-cancer tissues

Variable	Non-cancer tissues				Cancer tissues			
	n	ΔCt (Mean ± SD)	Normalized 2-ΔΔCt	*P*[1] value	*n*	ΔCt (Mean ± SD)	Normalized 2-ΔΔCt	*P*[1] value
The effect of HOTTIP rs17501292 genotypes to HOTTIP mRNA expression								
TT	10	12.32 ± 4.06	1 (0.60, 16.68)	Ref.	25	11.84 ± 3.87	1.39 (0.10, 20.39)	Ref.
TG	1	NA	NA	NA	2	15.30 ± 1.65	NA	0.735
GG	0	NA	NA	NA	0	NA	NA	NA
The effect of HOTTIP rs2067087 genotypes to HOTTIP mRNA expression								
GG	2	8.64 ± 2.06	1 (0.24, 4.17)	Ref.	5	11.66 ± 1.43	1 (0.37, 2.69)	Ref.
GC	5	12.25 ± 4.17	0.13 (0.01, 1.23)	0.253	13	11.85 ± 3.53	0.88 (0.08, 10.13)	0.101
CC	4	13.70 ± 3.86	0.03 (0.00, 0.86)	0.453	8	12.23 ± 5.42	0.67 (0.02, 28.84)	0.444
The effect of HOTTIP rs17427960 genotypes to HOTTIP mRNA expression								
CC	2	8.64 ± 2.06	1 (0.24, 4.17)	Ref.	6	12.47 ± 2.34	1 (0.20, 5.06)	Ref.
CA	5	11.55 ± 3.21	0.13 (0.01, 1.15)	0.254	13	11.88 ± 3.72	2.13 (0.04, 125.37)	0.063
AA	3	13.56 ± 4.71	0.03 (0.00, 0.86)	0.465	6	11.38 ± 5.88	1.51 (0.11, 19.84)	0.348
The effect of MALAT1 rs4102217 genotypes to MALAT1 mRNA expression								
GG	52	-2.25 ± 4.40	1 (0.05, 21.11)	Ref.	51	-0.98 ± 4.88	1 (0.03, 29.45)	Ref.
GC	16	-3.76 ± 3.60	2.85 (0.23, 34.54)	0.404	16	-2.79 ± 4.55	3.51 (0.15, 82.14)	0.742
CC	NA	NA	NA	NA	1	NA	NA	NA

[1]The statistical analysis for the effect of genotype to phenotype was used two-independent sample *t*-test, and for the combination of genotype to phenotype was used ANOVA analysis. NA: Not available.

LncRNAs function as ceRNAs to compete with mRNAs for access to miRNAs, which could regulate the expression of coding genes[5]. Most studies on lncRNA expression have focused on H19 and HOTAIR as well as other lncRNAs such as PRNCR1, *HOTTIP*, CCAT1, *CCAT2*, and *MALAT1*. *HOTTIP*, *CCAT2*, and *MALAT1* are all onco-lncRNAs, which have similar biological functions in promoting cell proliferation and invasion[23-29]. They can also promote HCC metastasis and epithelial-mesenchymal transition[30-33]. The *HOTTIP* gene is located in 7p15.2 and has three exons, the *CCAT2* gene is located in 8q24.21 and has one exon, and the *MALAT1* gene is located in 11q13.1 and has two exons. The most common SNPs reported for these genes are *HOTTIP* rs3807598, *CCAT2* rs6983267, and *MALAT1* rs619586. The first of these was found to be predictive of hematological toxicity in a three-way interaction pattern[34], and the latter two were indicated to be associated with platinum-based

chemotherapy response in lung cancer[11]. In this study, we found that SNPs in two exons (rs17501292 and rs2067087) and one intron (rs17427960) of the *HOTTIP* gene as well as an SNP in the promoter (rs4102217) of the *MALAT1* gene were associated with HCC risk; these variant alleles increased HCC risk in the range from 1.18- to 1.55-fold. These four SNPs are reported here for the first time to be associated with cancer risk. Concerning the commonly studied *HOTTIP* rs3807598 and *MALAT1* rs619586 SNPs, no significant associations with HCC risk were found in this study, which is consistent with the findings in a report by Liu[35]. In addition, we found that none of the *CCAT2* SNPs was associated with HCC risk. Following the identification of the possible significant SNPs, we further analyzed the relationship between the *HOTTIP* C-G-C-A haplotype of rs3807598-rs17501292-rs2067087-rs17427960 and HCC risk. The results showed an increase in HCC risk of 1.91-fold in those with this haplotype, and the OR value was greater than that for each SNP alone. Taking these findings together, it is newly indicated that the *HOTTIP* SNPs rs17501292, rs2067087, and rs17427960 and the *MALAT1* SNP rs4102217 have potential to be biomarkers for HCC risk.

Combined interaction analysis for multiple SNPs from different genes is more sensitive and powerful than one-dimensional SNP analysis[36]. For individual SNPs at single loci that were previously shown to have no or a weak effect on disease risk, an epistatic effect may appear when they are analyzed in combination[37]. One of the most significant findings in this study was the SNP-SNP interaction identified for the *HOTTIP* rs17501292-*MALAT1* rs619586 polymorphisms, which was confirmed by the epistatic effect analysis. In the main-effect analysis, *HOTTIP* rs17501292 had a weak effect, and *MALAT1* rs619586 had no effect on the risk of HCC. However, the pairwise analysis of these two in combination showed that they had an interactive effect on HCC risk. Subsequently, we analyzed the epistatic effect of these two SNPs and found that *MALAT1* rs619586 was associated with a decreased risk of HCC only in the presence of the *HOTTIP* rs17501292 TG+GG genotype. A similar epistatic effect between coding genes was also found in our previous study[38]. Further investigations are needed to verify our findings and the mechanism involved in the epistatic phenomenon.

In the prognostic analysis, we found no significant association of the studied SNPs with the overall survival of HCC patients. However, in the stratified analysis based on gender, we found that *MALAT1* rs591291 was associated with significantly better prognosis in the female subgroup. When stratified by HBV infection status, we found that patients carrying *HOTTIP* rs3807598 and *MALAT1* rs591291 variant genotypes had longer survival times. As some biomarkers are specific for certain subgroups and have potential to be used for the diagnosis or individualized therapy of specific subgroups[39], the above-mentioned polymorphisms could have value in predicting HCC prognosis for certain subgroups.

eQTL is an analysis in which the combination of mRNA expression and genotype data is applied to determine which variants are correlated with the transcription levels of genes[40]. We analyzed the SNPs potentially associated with HCC risk in our own data and then reanalyzed them in two public databases for the eQTL analysis. *HOTTIP* rs17501292 and rs2067087 are both located in exon 2 of this gene, while rs17427960 is in intron 2. In contrast, rs4102217 is located at -1255 bp of the *MALAT1* gene, within the promoter region. Among these four SNPs, we found that only the heterozygous genotype of intronic rs17427960 of the *HOTTIP* gene was associated with a higher lncRNA-*HOTTIP* expression level, with borderline significance. The public databases offered some supportive evidence for this from findings in other tissues, suggesting that rs4102217 in the *MALAT1* promoter is a functional SNP in 34 different tissues, such as pancreas and stomach, and that exonic rs17501292 of *HOTTIP* is a functional SNP in tibial artery tissue. In addition, some regulatory motifs, which were predicted by the bioinformatical software listed in Supplementary Figure 1, are transcription factors like PAX-4 and AP1. Thus, it is reasonable to assume that these SNPs could regulate certain motifs, leading to higher expression of oncogenic lncRNA and thus an elevation of HCC risk. However, further functional research is required to confirm this.

In summary, we found that the SNPs rs17501292, rs2067087, and rs17427960 in the *HOTTIP* gene, rs4102217 in the *MALAT1* gene, and a haplotype of *HOTTIP* increased the risk of HCC. In addition the SNPs *HOTTIP* rs3807598 and *MALAT1* rs591291 were associated with longer survival time in the HBV-negative subgroup.

ARTICLE HIGHLIGHTS

Research background

Genetic polymorphisms could be biomarkers for cancer risk and prognosis. Recent years, it was found that coding genes and non-coding genes had single nucleotide polymorphisms (SNPs). LncRNAs had important roles in the tumor incidence, progression, and prognosis. Thus, lncRNA polymorphisms had potential to be biomarkers for cancer precaution and prognostic prediction.

Research motivation

The aim of this study is to screen out the effective biomarkers for the hepatocellular cancer (HCC) risk and prognosis. The selected polymorphisms would have potential for the prediction of cancer risk and prognosis.

Research objectives

Five hundred and twenty-one patients of HCC and frequency matched 817 controls were studied for the cancer risk study. Among them, 351 patients for which the information was all available were recruited for the prognosis study. Then, 68 HCC specimens and corresponding samples from the noncancerous region were detected for the expression level study.

Research methods

For the risk and prognosis study, the samples were detected by the genomic DNA extracted and allele-specific PCR with KASPar reagents. The single nucleotide polymorphisms were selected by the Haploview software. The expression level study isolated the RNA isolated and then converted it to cDNA.

The SYBR based real-time PCR were adopted for the lncRNA expression.

Research results

We found the *HOTTIP* rs17501292, rs2067087, and rs17427960 SNPs increased HCC risk by 1.55-, 1.20-, and 1.18-fold under an allelic model, and the *MALAT1* rs4102217 SNP increased HCC risk by 1.32-fold under a dominant model. In addition, the two-way interaction of *HOTTIP* rs17501292 and *MALAT1* rs619586 polymorphisms decreased HCC risk and exhibited epistatic effects. In the survival analysis, the *HOTTIP* rs3807598 variant genotype showed significantly longer survival time in the hepatitis B virus (HBV)-negative subgroup, and *MALAT1* rs591291 showed an association with significantly better prognosis in the female and HBV-negative group. In this study, no significant effect in eQTL analysis was observed.

Research conclusions

Some specific *HOTTIP* and *MALAT1* SNPs have the potential to be biomarkers for HCC risk and prognosis.

Research perspectives

Screening SNPs could be valuable for the identification of biomarkers for HCC risk and prognosis. It could also be used for patient care, as there would be a cohort of patients who would benefit from screening using these positive SNPs.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy for the treatment of slow transit constipation in an aged population: A retrospective control study

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Abstract

AIM

To compare the efficacy, improved quality of life, and prognosis in patients undergoing either subtotal colonic bypass with antiperistaltic cecoproctostomy (SCBAC) or subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy (SCBCAC) for the treatment of slow transit constipation.

METHODS

Between October 2010 and October 2014, aged patients with slow transit constipation who were hospitalized and underwent laparoscopic surgery in our institute were

divided into two groups: the bypass group, 15 patients underwent SCBAC, and the bypass plus colostomy group, 14 patients underwent SCBCAC. The following preoperative and postoperative clinical data were collected: gender, age, body mass index, operative time, first flatus time, length of hospital stay, bowel movements (BMs), Wexner fecal incontinence scale, Wexner constipation scale (WCS), gastrointestinal quality of life index (GIQLI), numerical rating scale for pain intensity (NRS), abdominal bloating score (ABS), and Clavien-Dindo classification of surgical complications (CD) before surgery and at 3, 6, 12, and 24 mo after surgery.

RESULTS

All patients successfully underwent laparoscopic surgery without open surgery conversion or surgery-related death. The operative time and blood loss were significantly less in the bypass group than in the bypass plus colostomy group (P = 0.007). No significant differences were observed in first flatus time, length of hospital stay, or complications with CD > 1 between the two groups. No patients had fecal incontinence after surgery. At 3, 6, and 12 mo after surgery, the number of BMs was significantly less in the bypass plus colostomy group than in the bypass group. The parameters at 3, 6, 12, and 24 mo after surgery in both groups significantly improved compared with the preoperative conditions (P < 0.05), except NRS at 3, 6 mo after surgery in both groups, ABS at 12, 24 mo after surgery and NRS at 12, 24 mo after surgery in the bypass group. WCS, GIQLI, NRS, and ABS significantly improved in the bypass plus colostomy group compared with the bypass group at 3, 6, 12, and 24 mo after surgery (P < 0.05) except WCS, NRS at 3, 6 mo after surgery and ABS at 3 mo after surgery. At 1 year after surgery, a barium enema examination showed that the emptying time was significantly better in the bypass plus colostomy group than in the bypass group (P = 0.007).

CONCLUSION

Laparoscopic SCBCAC is an effective and safe procedure for the treatment of slow transit constipation in an aged population and can significantly improve the prognosis. Its clinical efficacy is more favorable compared with that of SCBAC. Laparoscopic SCBCAC is a better procedure for the treatment of slow transit constipation in an aged population.

Key words: Subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy; Subtotal colonic bypass with antiperistaltic cecoproctostomy; Minimally invasive surgery for treatment of constipation; Clinical efficacy; Slow transit constipation in an aged population

Core tip: Constipation is one of the most common gastrointestinal symptoms. From October 2010 to October 2014, we employed laparoscopic subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy (SCBCAC) to treat aged patients with constipation and conducted a retrospective control study in comparison with subtotal colonic bypass with antiperistaltic cecoproctostomy (SCBAC). From our study, we concluded that laparoscopic SCBCAC is an effective and safe procedure for the treatment of slow transit constipation in an aged population and can significantly improve the prognosis. Its clinical efficacy is more favorable compared with that of SCBAC.

Yang Y, Cao YL, Wang WH, Zhang YY, Zhao N, Wei D. Subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy for the treatment of slow transit constipation in an aged population: A retrospective control study. *World J Gastroenterol* 2018; 24(23): 2491-2500 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2491.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2491

INTRODUCTION

Constipation is one of the most common gastrointestinal symptoms. Generally, the incidence is 16% in females and 12% in males, but it affects more than 30% of the aged population[1] and seriously alters the life quality of patients. In terms of treatments for constipation[2], surgery is a common approach for treatment of intractable slow transit constipation (STC), especially for those with poor responses to conservative treatment. One of the two commonly used surgical approaches is total colectomy with ileorectal anastomosis (TC-IRA), which is widely used in the treatment of slow transit constipation because of its high cure rate[3-5]. Although constipation is alleviated, the main problem after surgery is the increased number of bowel movements (BMs), which causes some patients to suffer from abdominal pain, abdominal bloating, refractory diarrhea, loss of nutrients, and intestinal obstruction[5-8]. The other surgical approach is subtotal colectomy with cecorectal anastomosis (SCCRA)[9,10], which preserves the ileocecal valve and partial colon to be conducive to the absorption of water, electrolytes, bile salts, and vitamins and can alleviate severe postoperative diarrhea. More importantly, SCCRA is associated with a lower incidence of intestinal obstruction and can considerably improve the life quality of patients[11-14]. Based on different anastomosis sites, SCCRA is divided into two procedures: subtotal colectomy with isoperistaltic cecorectal anastomosis (SCICRA) and subtotal colectomy with antiperistaltic cecorectal anastomosis (SCACRA). SCICRA requires rotation of the ileocecal junction, which may easily lead to blood vessel torsion and poor blood circulation[9,15-19]. In contrast, SCACRA does not require rotation of the ileocecal junction and can avoid causing blood vessel torsion. Additionally, the function of the ileocecal junction is preserved. Thus, the SCACRA surgical method maintains the physiological anatomy[10,20-25]. Although the abovementioned methods have good efficacy in the treatment of slow transit

constipation, they are not suitable for aged patients or patients in poor physical condition because of the large wound produced and the length of the operation; these patients need non-surgical treatments. After long-term treatment with oral laxative agents, the patients become nonresponsive to these agents and have to undergo enema administration periodically to alleviate their constipation. Some patients cannot tolerate the suffering of constipation and have to choose ileostomy, which considerably impacts the quality of life of patients. In 2010, Yong-Gang Wang reported a subtotal colonic bypass with antiperistaltic cecoproctostomy (SCBAC) via an abdominal approach to treat slow transit constipation. In Wang's study, the average patient age was 51 years (range: 28-75 years). In patients who received 1-year follow-up, the alleviation rate of constipation was up to 75% (9/12)[26]. The procedure Wang used involved closing the distal portion of the cecum, after which side-side anastomosis was performed between the blinding end of the cecum and the rectum. In 2010, we performed four laparoscopic SCBACs in patients over 70 years old. After surgery, two patients experienced severe abdominal pain, abdominal bloating, rectal discomfort, and tenesmus. They did not respond to oral laxatives and required enemas for daily BMs. We examined these four patients with barium enemas and found barium retention in the excluded colon for 84 h in one case and 300 h in another. Therefore, we considered that the patient's postoperative symptoms may be related to the retention of indigested food and feces in the excluded colon. If the upper portion of the rectum was occluded with end-side anastomosis between the cecum and rectum, the excluded colon could become a closed loop. Thus, colonic mucus and fluid cannot be discharged, and observation of the excluded colon becomes impossible, which may cause delays in the detection and treatment of potential colonic lesions. From October 2010 to October 2014, we employed laparoscopic subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy (SCBCAC) to treat aged patients with constipation and conducted a retrospective control study in comparison with SCBAC.

MATERIALS AND METHODS

Patient selection

This was a two-phase study conducted in the Institute of Anal-Colorectal Surgery of PLA. From October 2010 to June 2012, 15 consecutive patients over 70 years old underwent laparoscopic SCBAC (LSCBAC); this group of patients was defined as the bypass group. From July 2012 to October 2014, 15 consecutive patients over 70 years old underwent laparoscopic SCBCAC (LSCBCAC); this group of patients was defined as the bypass plus colostomy group. Follow-ups were performed in these two groups of patients for more than 2 years. One patient in the bypass plus colostomy group was lost during follow-up. The preoperative examination included colonic transit test, defecography, colonoscopy, electromyography, anorectal manometry, and routine preoperative examination for colonic resection.

The surgical indications for this study are described as follows. Inclusion criteria: (1) The Rome III diagnosis criteria for constipation; (2) confirmed diagnosis of slow colon transit constipation (at least two positive colonic transit tests were recorded before surgery); (3) chronic (non-surgical treatment for more than 5 years), severe (WCS > 15), refractory (long-term dependence on high-dose laxative or application of enema) slow transit constipation; and (4) age ≥ 70 years. The exclusion criteria included: (1) American Society of Anesthesiologists (ASA) score > 3; (2) liver and kidney dysfunction; (3) patients with psychological symptoms or with history of mental illness, such as rectal abuse, vaginal abuse, *etc.*; (4) patients with obvious signs of outlet obstruction (such as frequent defecation, difficult defecation without dry feces, and anorectal dysfunction); (5) patients with a history of major abdominal surgery; (6) exclusion of organic colon disease; and (7) patients with life-threatening diseases, such as cancer.

Surgical procedure

Each procedure was performed by the same surgical team of the Institute of Anal-Colorectal Surgery of PLA. The patients were placed in the Trendelenburg position (≤ 15°) with legs apart. The pneumoperitoneum was maintained at 8 kPa or less. In the bypass plus colostomy group, laparoscopy was performed *via* five incisions. The five trocars were placed as follows: trocar 1 was placed at a site 0.5-1 cm above the umbilicus, trocar 2 was placed at the lateral margin of the left rectus abdominis muscle 4 cm below the umbilicus, trocar 3 was placed at the lateral margin of the left rectus abdominis muscle 2 cm above the umbilicus, and trocars 4 and 5 were placed at the mirror position of trocars 2 and 3 at the lateral margin of the right rectus abdominis muscle. The surgeon stood on the left side of the patient to mobilize the ileocecal junction and the ascending colon and to lower the ileocecal junction down to the pelvic inlet with preservation of the blood supply. A laparoscopic linear cutting stapler with a 45-mm green staple unit was used to transect the ascending colon at a site 2-3 cm distal to the ileocecal junction. Then, the appendix was excised. The surgeon then moved to the right side of the patient to dissect the lateral peritoneum of the sigmoid colon. At a site 4-6 cm above the peritoneal fold or at the level of the sacral promontory, a laparoscopic linear cutting stapler with a 45-mm green staple unit was used to transect the upper rectum after dissection of the rectal mesentery and ligation of the marginal arteries. The lower right abdominal incision was extended to the desired length. The head of a 29- to 33-mm circular stapler was placed in the bottom of the cecum. The shaft of the stapler was placed in the rectum *via* the anal canal to complete end-side anastomosis (end rectum to lateral cecum). The

ileocecal junction did not need rotation. The lower left abdominal incision was extended to desired length. The end of the rectal-sigmoid colon was used for colostomy *via* an extraperitoneal approach. At the end of the procedure, a drainage tube was placed in the Douglas' pouch.

In the bypass group, laparoscopy was performed *via* three incisions. The placements of trocars 1, 2, and 3 were the same as in the bypass plus colostomy group. The surgeon stood on the left side of the patient, and the surgical procedures were the same as those of the bypass plus colostomy group except for the rectal transection. The shaft of the stapler was placed in the rectum *via* the anal canal to complete side-side anastomosis between the right wall of the rectum and the cecum at the level above the rectal ampulla or sacral promontory. At the end of the procedure, a drainage tube was placed in the Douglas' pouch.

Patient data collection

The following statistical data were collected: (1) Patient sex, age, and body mass index (BMI); (2) surgical parameters (operative time and blood loss); and (3) postoperative recovery (first flatus time, length of hospital stay, and postoperative complications). The following clinical data were collected before surgery and 3, 6, and 12 mo after surgery: the number of daily BMs and the Wexner incontinence scale (WIS, on a scale of 0-20, in which 0 represents the best and 20 represents complete incontinence)[4]. The following clinical data were collected before surgery and 3, 6, 12, and 24 mo after surgery: The Wexner constipation scale[27] (WCS, on a scale of 0-30 in which the higher score represents more severe constipation; the scores of healthy individuals are less than 8), the gastrointestinal quality of life index[28] (GIQLI, on a scale of 0-144 in which (125.80 ± 13.00) represents the index in healthy population), abdominal pain intensity indicated by the numerical rating scale (0-10)[29] (NRS, 0-3: Mild pain and no impact on sleep; 4-6: Moderate tolerable pain and mild impact on sleep; 7-10: Severe pain and serious impact on sleep), and the abdominal bloating score (ABS, the score is inferred from the GIQLI scoring table, from 0-4: 0 = absent; 1 = occasionally; 2 = sometimes; 3 = most of the time; 4 = all the time). Symptoms with scores > 1 were defined as surgery-related abdominal pain and bloating, and symptoms with scores ≥ 3 were defined as severe postoperative frequent abdominal pain and bloating. The Clavien-Dindo classification[30] was used to assess surgical complications. The complications defined as class II or above were studied. All postoperative data were obtained from the questionnaire by clinical visit or telephone follow-up. This study began in October 2012.

Statistical analysis

We compared the postoperative parameters at 3, 6, 12, and 24 mo after surgery of the two groups, including the WCS, ABS, GIQLI, and NRS with preoperative parameters. We studied the variations in parameters among patients in each group. We also compared the postoperative parameters between the two groups at 3, 6, 12, and 24 mo after surgery, including the WIS, WCS, ABS, GIQLI, NRS, and BM. Thus, the effects of two different surgical methods for the treatment of STC patients were evaluated.

The variables were expressed as mean ± standard deviation (SD). T-tests were used for the comparison of paired data at different time points within each group. For the comparison of data between the two groups, independent samples *t*-test and Fisher's exact test were applied. For the comparison of postoperative functional recovery (WIS, WCS, ABS, GIQLI, NRS, and BM) between groups, analysis of covariance was applied. $P < 0.05$ was considered statistically significant. All data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Basic information and preoperative data

Patient characteristics were not significantly different between the two groups. Both groups predominantly consisted of females, with ages ranging from 70 to 80 years. The average age was 74.86 ± 3.42 in the bypass plus colostomy group and 74.73 ± 3.11 in the bypass group. All patients in both groups suffered from severe constipation before surgery. The preoperative WCS scores were 16.86 ± 1.56 and 16.93 ± 1.16 in the bypass plus colostomy group and the bypass group, respectively. The preoperative GIQLI scores were very low in both groups (64.00 ± 3.51 and 63.20 ± 2.40 in the bypass plus colostomy group and the bypass group, respectively). No patient in either group experienced fecal incontinence but somewhat suffered from abdominal pain and bloating (Table 1).

Surgical data and postoperative results

All patients successfully underwent laparoscopic surgery without open surgery conversion or surgery-related death. The operative times were short in both groups (42.67 ± 3.35 min in the bypass plus colostomy group and 36.56 ± 4.06 min in the bypass group). However, the operative time was significantly longer in the bypass plus colostomy group than in the bypass group ($P < 0.001$). The blood loss was negligible in both groups (14.43 ± 3.11 mL in the bypass plus colostomy group and 11.13 ± 2.93 mL in the bypass group). However, the blood loss was significantly less in the bypass group than in the bypass plus colostomy group ($P = 0.007$). No significant differences were observed in first flatus time or length of hospital stay between the two groups ($P = 0.317$ and $P = 0.644$, respectively). We compared each complication of Clavien-Dindo > 1 and did not note differences between the groups ($P = 0.007$) (Table 1). No anastomotic leakage was reported in either group,

Table 1 Clinical characteristics of patients (mean ± SD)

			Bypass plus colostomy group (n = 14)	Bypass group (n = 15)	P value
Basic information	Sex	M/F	6/8	5/10	0.710
	Age	yr	74.86 ± 3.42	74.73 ± 3.11	0.919
	BMI	kg/m^2	20.03 ± 1.09	20.27 ± 1.39	0.612
Preoperative data	WCS	(0-30)	16.86 ± 1.56	16.93 ± 1.16	0.882
	GIQLI	(0-144)	64.00 ± 3.51	63.20 ± 2.40	0.477
	ABS	(0-4)	2.71 ± 0.73	2.40 ± 0.63	0.224
	NRS	(0-10)	3.00 ± 1.04	2.87 ± 1.30	0.764
Operative and postoperative data	Operative time	min	42.67 ± 3.35	36.86 ± 4.06	< 0.001
	Blood loss	mL	14.43 ± 3.11	11.13 ± 2.95	0.007
	First flatus time	d	1.86 ± 1.03	2.20 ± 0.78	0.317
	Hospital stay	d	14.00 ± 1.66	13.67 ± 2.13	0.644
	Morbidity (Dindo > I)	n (%)	1 (7.14)	1 (6.67)	0.960

BMI: Body mass index; WCS: Wexner constipation scale; GIQLI: Gastrointestinal quality of life index; ABS: Abdominal bloating score; NRS: Numerical rating scale.

but one case of pneumonia was reported in each group. Both cases of pneumonia were cured. No patients had fecal incontinence after surgery. At 3, 6, and 12 mo after surgery, the WIS was significantly better, and the number of BMs was significantly less in the bypass plus colostomy group than in the bypass group.

Functional recovery

Functional recovery compared at different time points within the same group: WCS and GIQLI significantly improved (P < 0.001) at 3, 6, 12, and 24 mo after surgery in both groups. In the bypass plus colostomy group, NRS significantly improved at 12 and 24 mo after surgery (P < 0.001); ABS significantly improved (P < 0.001) at 3, 6, 12, and 24 mo after surgery. In the bypass group, NRS did not improve at 3, 6, 12, and 24 mo after surgery; ABS significantly improved (P = 0.003) at 3 and 6 mo but did not improve at 12 and 24 mo after surgery (P = 0.207 and P = 0.670, respectively) (Table 2).

Functional recovery compared between groups: At 3, 6, 12, and 24 mo after surgery, WCS, GIQLI, NRA, and ABS were compared between the two groups. WCS and NRS remained unimproved at 3 and 6 mo after surgery, and ABS remained unchanged at 3 mo after surgery. Additional above-noted parameters were significantly better in the bypass plus colostomy group than in the bypass group at each time point. These improvements continued over the time course, as shown in Figure 1 and Table 3.

At 1 year after surgery, barium enema examinations were performed in all patients of both groups. The barium emptying times were 22.71 ± 4.41 h and 113.60 ± 110.53 h in the bypass plus colostomy group and the bypass group, respectively. The former group was significantly better than the latter group (P = 0.007). In the bypass group, barium emptying time ≥ 72 h was seen in eight (53.33%) patients. In contrast, the longest barium emptying time was 30 h in the bypass plus colostomy group and did not exceed 72 h (P = 0.002).

DISCUSSION

Primarily, constipation occurs in the aged population and shows increased incidence and severity with aging. Aged patients over 70 years often have varying degrees of accompanying cardiovascular and cerebrovascular diseases and cannot tolerate some major surgeries. In our study, the preserved length of the ileocecal junction was determined in reference to Wei's study[31] in which the lengths of the preserved cecum were 2-3 cm distal to the upper edge of the ileocecal junction. All surgeries in the 29 patients were successful. The surgical procedures were the same in both groups except for the cecorectal anastomosis, for which side-side anastomosis was used in the bypass group, while end-side anastomosis and colostomy were used in the bypass plus colostomy group. The preoperative characteristics of the patients were not significantly different between the two groups. Water and fluid diet could be started 24 h after surgery. No obvious abdominal pain was reported after surgery, and the signs of early recovery of intestinal function were noted. The follow-up data showed there was no intestinal obstruction due to adhesions that required surgery.

The results obtained in this study for the treatment of slow transit constipation in an aged population with SCBCAC are very satisfactory. The quality of life has been improved significantly after operations in all the patients. This indicates that these two surgeries have advantages, including minimal trauma, fast recovery, and safe and feasible procedures. There are some explanations for these good results. The first is that we selected the patients strictly before operation. Rigorous psychological assessment is needed before surgery in order to eliminate the patients with psychological symptoms or with history of mental illness. Also, a careful physiologic assessment is necessary to eliminate other causes of constipation, such as organic colon disease, outlet

Table 2 Preoperative and postoperative functional recovery results (mean ± SD)

	Bypass plus colostomy group (n = 14)				Bypass group (n = 15)			
	Preoperative	Postoperative		P value	Preoperative	Postoperative		P value
WCS	16.86 ± 1.56	3 mo	2.71 ± 2.30	< 0.001	16.93 ± 1.16	3 mo	4.33 ± 3.83	< 0.001
		6 mo	2.64 ± 2.50	< 0.001		6 mo	5.07 ± 4.06	< 0.001
		12 mo	2.36 ± 2.13	< 0.001		12 mo	6.40 ± 5.16	< 0.001
		24 mo	1.86 ± 1.46	< 0.001		24 mo	6.60 ± 5.42	< 0.001
GIQLI	64.00 ± 3.51	3 mo	106.57 ± 5.79	< 0.001	63.20 ± 2.40	3 mo	88.27 ± 12.26	< 0.001
		6 mo	114.50 ± 7.59	< 0.001		6 mo	95.13 ± 14.87	< 0.001
		12 mo	119.79 ± 8.24	< 0.001		12 mo	97.60 ± 18.38	< 0.001
		24 mo	122.21 ± 6.85	< 0.001		24 mo	98.47 ± 18.09	< 0.001
ABS	2.71 ± 0.73	3 mo	1.29 ± 0.83	< 0.001	2.40 ± 0.63	3 mo	1.53 ± 0.83	0.003
		6 mo	0.86 ± 0.77	< 0.001		6 mo	1.67 ± 0.82	0.003
		12 mo	0.86 ± 0.66	< 0.001		12 mo	2.07 ± 0.88	0.207
		24 mo	0.79 ± 0.70	< 0.001		24 mo	2.27 ± 1.16	0.670
NRS	3.00 ± 1.04	3 mo	2.50 ± 1.29	0.187	2.87 ± 1.30	3 mo	3.27 ± 1.34	0.320
		6 mo	2.21 ± 1.47	0.094		6 mo	3.53 ± 2.00	0.126
		12 mo	1.14 ± 0.86	< 0.001		12 mo	3.93 ± 2.92	0.123
		24 mo	1.07 ± 0.62	< 0.001		24 mo	4.07 ± 3.04	0.105

WCS: Wexner constipation scale; GIQLI: Gastrointestinal quality of life index; ABS: Abdominal bloating score; NRS: Numerical rating scale.

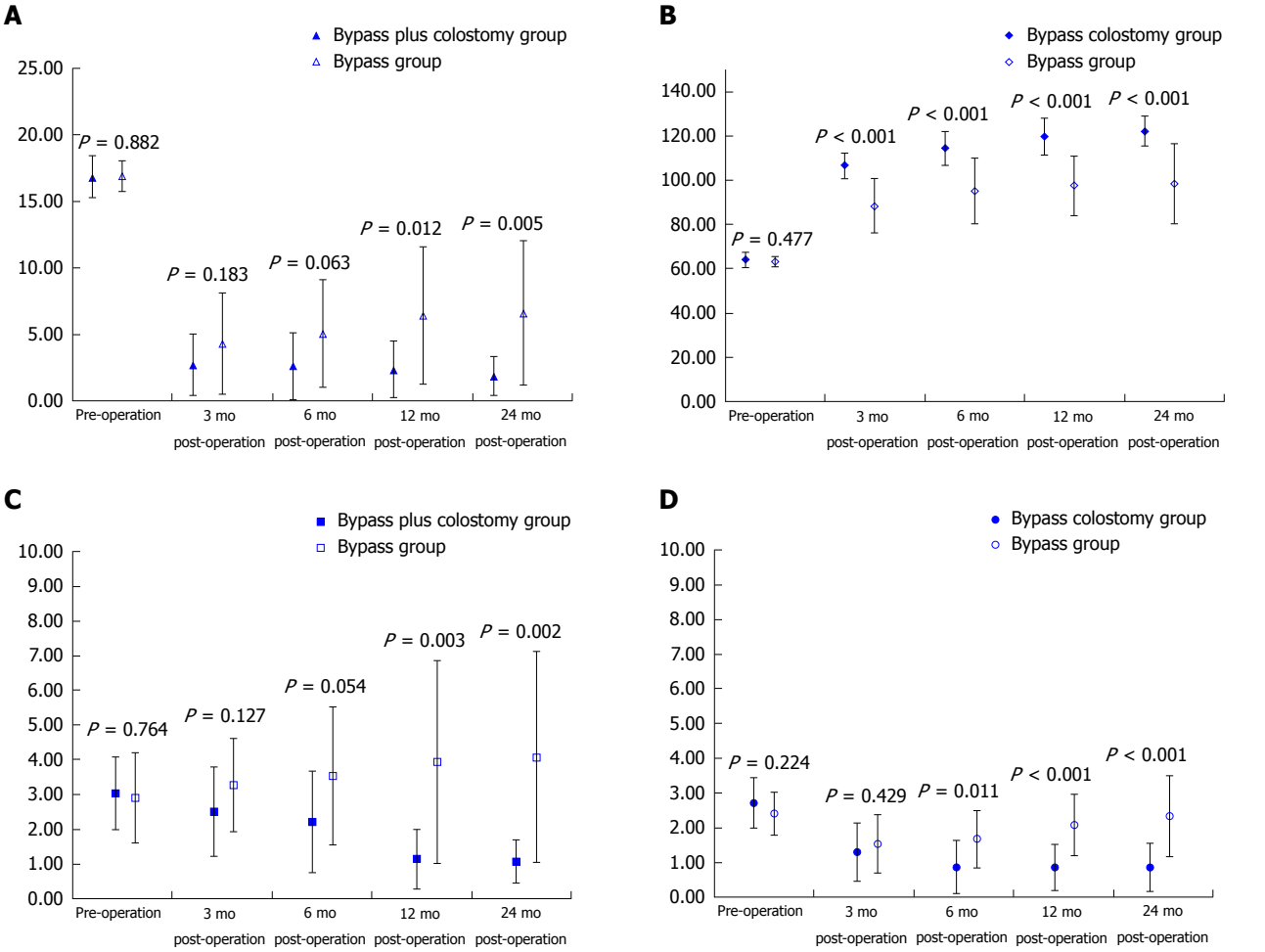

Figure 1 X-axis: Preoperative and postoperative time points. A: Y-axis: WCS scores; ▲: Mean of WCS in the bypass plus colostomy group; △: Mean of WCS in the bypass group; │: 95% confidence interval of WCS. B: Y-axis: GIQLI scores; ◆: Mean of GIQLI in the bypass plus colostomy group; ◇: Mean of GIQLI in the bypass group; │: 95% confidence interval of GIQLI. C: Y-axis: NRS scores; ■: Mean of NRS in the bypass plus colostomy group; □: Mean of NRS in the bypass group; │: 95% confidence interval of NRS. D: Y-axis: ABS scores; ●: Mean of ABS in the bypass plus colostomy group; ○: Mean of ABS in the bypass group; │: 95% confidence interval of ABS. WCS: Wexner constipation scale; GIQLI: Gastrointestinal quality of life index; ABS: Abdominal bloating score; NRS: Numerical rating scale.

Table 3 Postoperative functional recovery comparison between the two groups (mean ± SD)

	Time (postoperative)	Bypass plus colostomy group (n = 14)	Bypass group (n = 15)	P value
Barium emptying times	12 mo	22.71 ± 4.41	113.60 ± 110.53	0.007
BMs	3 mo	4.07 ± 1.90	5.60 ± 1.24	0.016
	6 mo	3.21 ± 0.89	4.20 ± 1.47	0.038
	12 mo	2.43 ± 0.85	3.60 ± 1.35	0.010
WIS	3 mo	4.14 ± 1.41	5.60 ± 1.60	0.015
	6 mo	1.86 ± 1.29	3.87 ± 1.55	0.001
	12 mo	1.36 ± 0.63	3.53 ± 2.00	0.001
WCS	3 mo	2.71 ± 2.30	4.33 ± 3.83	0.183
	6 mo	2.64 ± 2.50	5.07 ± 4.06	0.063
	12 mo	2.36 ± 2.13	6.40 ± 5.16	0.012
	24 mo	1.86 ± 1.46	6.60 ± 5.42	0.005
GIQLI	3 mo	106.57 ± 5.79	88.27 ± 12.26	< 0.001
	6 mo	114.50 ± 7.59	95.13 ± 14.87	< 0.001
	12 mo	119.79 ± 8.24	97.60 ± 18.38	< 0.001
	24 mo	122.21 ± 6.85	98.47 ± 18.09	< 0.001
ABS	3 mo	1.29 ± 0.83	1.53 ± 0.83	0.429
	6 mo	0.86 ± 0.77	1.67 ± 0.82	0.011
	12 mo	0.86 ± 0.66	2.07 ± 0.88	< 0.001
	24 mo	0.79 ± 0.70	2.27 ± 1.16	< 0.001
NRS	3 mo	2.50 ± 1.29	3.27 ± 1.34	0.127
	6 mo	2.21 ± 1.47	3.53 ± 2.00	0.054
	12 mo	1.14 ± 0.86	3.93 ± 2.92	0.003
	24 mo	1.07 ± 0.62	4.07 ± 3.04	0.002

BMs: Bowel movements; WIS: Wexner fecal incontinence scale; WCS: Wexner constipation scale; GIQLI: Gastrointestinal quality of life index; ABS: Abdominal bloating score; NRS: Numerical rating scale.

obstruction syndrome, mixed constipation, or small intestinal dysfunction. The other possible reason for the results is the innovation in operative procedures. The procedure of SCBCAC can be manipulated simply and has characteristic features of less invasion and good effect. It also should intuitively require shorter operation time and less risk of contamination during surgery so that the aged population could be well-tolerated and compatible with this procedure.

We found that WCS and GIQLI at 3, 6, 12, and 24 mo after surgery significantly improved compared with the values before surgery in both groups. In the bypass plus colostomy group, ABS significantly improved at 3, 6, 12, and 24 mo after surgery compared with that before surgery. Compared with NRS before surgery, NRS improvement was not noted at 3 and 6 mo but was evident at 12 and 24 mo after surgery. In the bypass group, NRS did not improve at 3, 6, 12, and 24 mo after surgery compared with that before surgery. ABS improvement was seen at 3 and 6 mo but disappeared at 12 and 24 mo after surgery. These results suggest that the efficacy of the bypass plus colostomy is better than that of the bypass group. The reasons for these observations may be that SCBAC cannot improve but can actually worsen symptoms of abdominal pain and bloating.

Based on changes in WIS and BM, we noted that the number of BMs increased somewhat in both groups; however, the movements appeared to decrease over time. These results indicate that the number of BMs at 6 or 12 mo after surgery was lower in the bypass plus colostomy group than in the bypass group. WIS was relatively low in both groups 3 mo after surgery and appeared to decrease over time. Obviously, the WIS improvements at 3, 6, and 12 mo after surgery were significantly better in the bypass plus colostomy group than in the bypass group. The above-noted results suggest that the isolated bypass surgery cannot improve the symptoms of abdominal pain and bloating but can worsen the symptoms of rectal discomfort and increase the number of BMs.

In terms of the changes in WCS, GIQLI, ABS, and NRS at 3, 6, 12, and 24 mo after surgery, we noted that WCS was not significantly different at 3 and 6 mo after surgery between the two groups but that it improved in the bypass plus colostomy group and worsened in the bypass group overtime. WCS at 12 and 24 mo after surgery was better in the bypass plus colostomy group than in the bypass group. In the bypass plus colostomy group, GIQLI at 3 mo after surgery significantly improved and continued to improve over time, eventually reaching the average level of the healthy population. In the bypass group, GIQLI improved without significance. At 6, 12, and 24 mo after surgery, GIQLI was significantly better in the bypass plus colostomy group than in the bypass group. ABS and NRS were not different at 3 mo after surgery, but these parameters continued to improve in the bypass plus colostomy group but conversely worsened in the bypass group over time. At 12 and 24 mo after surgery, ABS and NRS became significantly different between the two groups. In terms of the incidence rates of postoperative abdominal pain and bloating, the incidence rates of severe abdominal bloating were 0 *vs* 46.67% (7/15) between the bypass

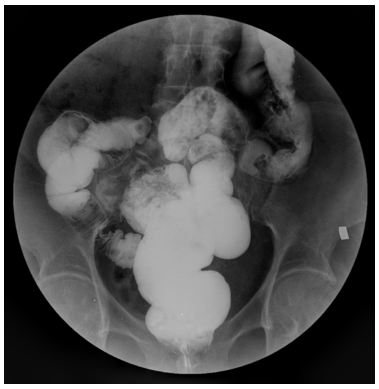

Figure 2 Barium enema examination of a patient in the bypass group at 1 year after surgery.

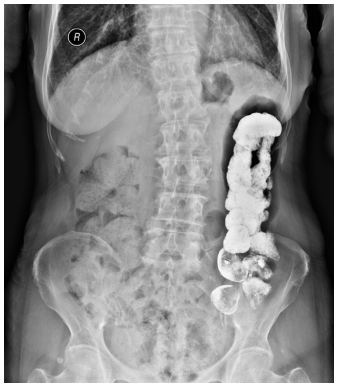

Figure 3 The longest emptying time of a patient in the bypass group: 360 h after barium enema examination at 1 year after surgery.

plus colostomy group and the bypass group, and the incidence rates of severe abdominal pain were 0 *vs* 40% (6/15), respectively, between the bypass plus colostomy group and the bypass group. These results suggest that bypass plus colostomy has more advantages in improving the symptoms of constipation, relieving abdominal pain and bloating, and improving the quality of life in aged patients.

What are the causes of abdominal pain, abdominal bloating, rectal discomfort, and increased BMs after surgery? To address these questions, we performed barium enema examinations in these patients at 1 year after surgery and compared the barium emptying times between the two groups. The barium emptying time was significantly shorter in the bypass plus colostomy group than in the bypass group. Moreover, the barium retention sites were in the excluded colon rather than in the small intestine. Further analysis of these data revealed that the longest emptying times were 30 h and 360 h in the bypass plus colostomy group and the bypass group, respectively (shown in Figures 2 and 3). Moreover, the barium emptying times were > 72 h in eight of 15 patients of the bypass group. Therefore, we postulated that postoperative abdominal bloating may be caused by undigested food entering the excluded colon, which produces gas and that postoperative abdominal pain may be caused by food-residue-stimulated peristalsis or even intestinal spasm. The feces in the excluded colon will cause a desire for defecation and rectal discomfort, but the amount of defecation each time is small, and the patient experiences a feeling of incomplete defecation. These symptoms reduce patient quality of life. To this end, we recommend using subtotal colonic bypass plus colostomy rather than an isolated bypass of the colon for the treatment of refractory constipation in aged patients.

Of course, there is no denying that colostomy may bring a little inconvenience to the patients' daily life compared with healthy people, but unlike other permanent colostomy, the colostomy in SCBCAC does not need to excrete a large amount of stool every day. In our study, the healing of the abdominal wall stoma was favorable. A small amount of intestinal fluid or mucus was drained every 1-3 d, but the drainage amount gradually decreased over time. No ulcers or hemorrhages were seen in the skin around the stoma because no feces were discharged from it. The daily life of patients was not negatively affected. Obviously, colostomy for benign disease did not influence quality of life in aged population. However, this could not be accepted for a younger patient population. Also, there is a problem that tumor occurrence might be increased in the nonfunctional colon and further research is needed to confirm this.

This work is a retrospective single-center study and has certain limitations. We will further develop a multicenter randomized controlled study. Meanwhile, we will expand the sample size and continue long-term follow-up to further evaluate the efficacy of the subtotal colonic bypass plus colostomy.

Laparoscopic SCBCAC is an effective procedure for the treatment of slow transit constipation and is particularly suitable for aged people in poor physical condition who are not suitable for subtotal colonic resection. The efficacy of laparoscopic SCBCAC is superior to that of SCBAC in the aged population.

ARTICLE HIGHLIGHTS

Research background

Constipation affects more than 30% of the aged population and seriously alters the life quality of patients. In terms of treatments for constipation, surgical treatment is a common approach for treatment of intractable slow transit constipation, especially for those with poor responses to conservative treatment. This study offers a better procedure for the treatment of slow transit constipation in an aged population.

Research motivation

Although the current surgical methods have good efficacy in the treatment of slow transit constipation, they are not suitable for aged patients or patients in poor physical condition because of the large wound produced and the length of the operation; these patients need non-surgical treatments. After long-term treatment with oral laxative agents, patients become nonresponsive to these agents and have to undergo enema administration periodically to alleviate their constipation. Some patients cannot tolerate the suffering of constipation and

have to choose ileostomy.

Research objectives

The main aim of this study is to compare the efficacy, improved quality of life, and prognosis in patients undergoing either subtotal colonic bypass with antiperistaltic cecoproctostomy (SCBAC) or subtotal colonic bypass plus colostomy with antiperistaltic cecoproctostomy (SCBCAC) for the treatment of slow transit constipation.

Research methods

Aged patients between October 2010 and October 2014, who had slow transit constipation, were hospitalized and underwent laparoscopic surgery in our institute and were divided into two groups: the bypass group and the bypass plus colostomy group. The following preoperative and postoperative clinical data were collected: gender, age, body mass index, operative time, first flatus time, length of hospital stay, bowel movements (BMs), Wexner fecal incontinence scale, Wexner constipation scale (WCS), gastrointestinal quality of life index (GIQLI), numerical rating scale for pain intensity (NRS), abdominal bloating score (ABS), and Clavien-Dindo classification of surgical complications (CD) before surgery and at 3, 6, 12, and 24 mo after surgery.

Research results

All patients successfully underwent laparoscopic surgery without open surgery conversion or surgery-related death. The operative time and blood loss were significantly less in the bypass group than in the bypass plus colostomy group. No significant differences were observed in first flatus time, length of hospital stay, or complications with CD > 1 between the two groups. No patients had fecal incontinence after surgery. At month 3, 6, and 12 after surgery, the number of BMs was significantly less in the bypass plus colostomy group than in the bypass group. The parameters at month 3, 6, 12, and 24 after surgery in both groups significantly improved compared with the preoperative conditions, except for NRS at month 3 and 6 after surgery in both groups, ABS at month 12 and 24 after surgery, and NRS at month 12 and 24 after surgery in the bypass group. WCS, GIQLI, NRS, and ABS significantly improved in the bypass plus colostomy group compared with the bypass group at month 3, 6, 12, and 24 after surgery except WCS, NRS at month 3, 6 after surgery and ABS at month 3 after surgery. At 1 year after surgery, a barium enema examination showed that the emptying time was significantly better in the bypass plus colostomy group than in the bypass group.

Research conclusions

We draw a conclusion from this study that laparoscopic SCBCAC is an effective and safe procedure for the treatment of slow transit constipation in an aged population and can improve the prognosis significantly. Its clinical efficacy is more favorable compared with that of SCBAC. Laparoscopic SCBCAC is a better procedure for the treatment of slow transit constipation in the aged population.

Research perspectives

This work is a retrospective single-center study. We will further develop a multicenter randomized controlled study. Meanwhile, we will expand the sample size and continue long-term follow-up to evaluate further efficacy of the subtotal colonic bypass plus colostomy.

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ORIGINAL ARTICLE

Retrospective Study

Transarterial embolization and low-dose continuous hepatic arterial infusion chemotherapy with oxaliplatin and raltitrexed for hepatocellular carcinoma with major portal vein tumor thrombus

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Author contributions: Zhu LZ and Qian HL designed and performed this study; Zhu LZ analyzed the data; Xu S wrote the paper; Zhu LZ revised the manuscript for final submission.

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Abstract

AIM

To determine the efficacy and safety of transarterial embolization and low-dose continuous hepatic arterial infusion chemotherapy with oxaliplatin and raltitrexed in hepatocellular carcinoma (HCC) with major portal vein tumor thrombus (MPVTT).

METHODS

Eighty-six patients with MPVTT accepted routine embolization. The catheter was kept in the hepatic artery and oxaliplatin (50 mg in 250 mL of glucose) was infused by pump for 4 h, followed by raltitrexed (2 mg in 100 mL of 0.9% saline) infusion by pump for the next 1 h. The efficacy and safety were evaluated after

the transarterial chemoembolization (TACE).

RESULTS

Full or partial embolization was achieved in 86 cases, where all the cases received low dose continuous hepatic arterial infusion chemotherapy. Complete responses (CRs), partial responses (PRs), stable disease (SD), and disease progression (PD) for intrahepatic disease were observed in 0, 45, 20, and 21 patients, respectively. The 1-, 2-and 3-year overall survival rates of the 86 patients were 40.7%, 22.1%, and 8.1% respectively, and the median survival time was 8.7 mo. Complication was limited.

CONCLUSION

TACE with low dose continuous hepatic arterial infusion of oxaliplatin and raltitrexed could be an option in MPVTT patient; it was shown to be effective in patients with advanced HCC with MPVTT with less toxicity.

Key words: Transarterial embolization; Oxaliplatin; Major portal vein tumor thrombus; Raltitrexed; Continuous hepatic arterial infusion chemotherapy

Core tip: We analyzed the pharmacokinetic and pharmacodynamic characteristics of continuous hepatic arterial infusion of oxaliplatin and raltitrexed to aid interventional radiologist in determining a more accurate infusion protocol. The clinical protocol based on the pharmacokinetics study in a swine model and the pharmacodynamics study in tumor cell lines was shown to be effective and safe in hepatocellular carcinoma with main portal vein tumor thrombus.

Zhu LZ, Xu S, Qian HL. Transarterial embolization and low-dose continuous hepatic arterial infusion chemotherapy with oxaliplatin and raltitrexed for hepatocellular carcinoma with major portal vein tumor thrombus. *World J Gastroenterol* 2018; 24(23): 2501-2507 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2501.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2501

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third most common cause of cancer-related death worldwide[1,2], particularly in Asian countries[3]. Recent studies have indicated that the overall clinical survival of HCC remains extremely poor, especially among patients with invasion of the portal vein branches[4]. Portal vein tumor thrombosis (PVTT) is a strong predictor of mortality in patients with HCC[5] and is usually associated with an unsatisfactory prognosis[6]. The median survival time is only approximately 2-4 mo compared with 10-24 mo among those without PVTT[7]. When a thrombus involves the main portal vein, the prognosis is much worse than in cases in which a thrombus involves a branch of the portal vein[8].

Treatment of advanced HCC with major portal vein tumor thrombosis (MPVTT) remains a challenge. MPVTT can increase the risk of wide tumor transmission and also increase the pressure of the portal vein (especially when accompanied with arterial portal vein shunt), which results in acute variceal hemorrhaging, intractable ascites, hepatic encephalopathy, and failure. All of these conditions limit treatment choices.

It is well known that transarterial chemotherapy can achieve high local concentrations. Many patients who cannot endure systemic chemotherapy can benefit from transarterial chemoembolization (TACE). We have adopted continuous hepatic arterial infusion chemotherapy technique in our center for the treatment of colon cancer liver metastasis and have demonstrated its safety and advantage[9]. However, PVTT patients cannot endure a regular dose of chemotherapy due to its high risk and high complexity, and we need a more effective and safe protocol that uses the smallest dose possible to provide a sufficient effect while limiting toxicity.

When we sought to create a protocol for transarterial infusion chemotherapy (TAI), we needed to determine a total dose and infusion time, but we first needed to solve following problems: (1) How does a certain dose and infusion time influence the concentration in local tissue? (*i.e.*, pharmacokinetics)? (2) How do high-concentration drugs interact with the tumor (*i.e.*, pharmacodynamics)? And (3) What are the optimum time and concentration for the application of a certain chemotherapy drug, and how can these parameters be realized in the clinic?

We firstly performed a pharmacokinetics study in a swine model and pharmacodynamics studies in different tumor cell lines to learn the real-time drug concentration in local tissue and how tumor cells respond under such an environment during continuous hepatic arterial infusion chemotherapy. Based on our findings, we determined the suitable concentration and infusion time for a certain agent, designed a protocol based on the above mentioned studies, and observed its efficacy and safety for patients with MPVTT.

MATERIALS AND METHODS

Patients

This was a retrospective study. All patients were admitted to our hospital between May 2010 and March 2017. A total of 86 consecutive patients with unresectable advanced HCC and major PVTT received TACE. The diagnoses of HCC were established using pathology in 64 patients, and the diagnoses were established based on two types of typical HCC imaging, *i.e.*, computed tomography (CT) and magnetic resonance imaging

Table 1 Characteristics of the patients with major portal vein tumor thrombosis

MPVTT characteristics	Portal trunk + left branch	Portal trunk + right branch	Portal trunk + SMV or SV	Portal trunk + IVC
n	27	36	12	11
Gender				
Male	27	30	8	8
Female	0	6	4	3
Age	35-84	33-72	42-70	37-74
Child-Pugh grade				
Child A	6	6	3	3
Child B	17	22	5	6
Child C	4	8	4	2
Tumor Size				
5-10 cm	15	16	8	7
> 10 cm	12	20	4	4
Gastric esophageal varices	11	30	7	7
Splenomegaly	15	32	7	6
Hypersplenism	12	26	5	3
Ascites	10	22	7	4
Hepatic artery-portal vein shunt	10	15	3	7

MPVTT: Main portal vein tumor thrombus.

(MRI), plus a serum alpha-fetoprotein (AFP) level of more than 400 ng/mL in 30 patients.

All patients were categorized as American Joint Committee on Cancer stage IIIa (6th edition)[10]. Portal vein thrombosis was evaluated according to criteria of the Liver Cancer Group of Japan[11].

The baseline data are presented in Table 1. Most of the patients were positive for hepatitis B virus (HBV) infection. Seventy-eight patients were male, and 16 were female. Forty-four (46.8%) patients had a tumor larger than 10 cm. Etiologies of HBV and HCV infection were identified in 72 and four cases, respectively. The age range was 31-84 years (mean: 53.7 years). The pre-treatment evaluations consisted of a complete history and physical examination, blood cell count, liver and renal panels, AFP value assessment, and a tri-phase CT scan or dynamic MRI of the liver. All patients had an ECOG performance status ≤ 2, a serum total bilirubin ≤ 3.0 mg/L, a serum aminotransferase ≤ 100 IU/L, a white blood cell count ≥ 3000/μL, a hemoglobin level ≥ 8 g/dL, and a platelet level ≥ 7.5 × 10^4/μL or corrected to this level *via* embolization of the spleen. Sixteen patients had gastrointestinal bleeding, and 46 patients had ascites upon admission.

Protocol for the continuous hepatic arterial infusion of oxaliplatin and raltitrexed

After routine embolization, we kept the catheter in the segmental artery and confirmed its proper position *via* fluorescence imaging. When the patient was returned to the ward, 50 mg oxaliplatin in 250 mL of glucose was infused with a pump over 4 h. Then, 2 mg raltitrexed in 100 mL of 0.9% normal saline was infused with a pump over the next 1 h. Ondansetron was used to prevent vomiting.

The treatment protocol began with 1-11 sessions of TACE (average interval: 3.2 mo). The patients were followed up every 1-3 mo to check whether new tumor lesions had developed in the liver. When lesions were detected, TACE was performed again to control the intrahepatic lesions.

Evaluation

The acute and late toxicities from the treatments were graded according to the NCI-CTCAE version 4.0[12] Monthly evaluations of the responses to TACE were recommended.

The responses were defined using the mRECIST criteria[13] based on an enhanced CT and MRI of the liver.

Statistical analysis

SPSS version 19.0 (SPSS Inc., Chicago, IL, United States) for Windows was used for the data analysis. Overall survival (OS) was calculated from the date of HCC diagnosis until death or until the date of the last follow-up visit for all patients who were still alive.

RESULTS

Full embolization was achieved in 68 cases, and partial embolization was achieved in 18 cases. For 86 patients who received embolization, the catheter was kept in the hepatic artery and them low-dose continuous hepatic arterial infusion chemotherapy was administered. Radiologically complete responses (CRs), partial responses (PRs), stable disease (SD), and disease progression (PD) of the intrahepatic disease were observed in 0, 45, 20, and 21 patients, respectively. The following time was 1-55 mo, the 1-, 2- and 3-year overall survival rates of the 86 patients were 40.7%, 22.1% and 8.1%, respectively, and the median survival time was 8.7 mo. Moreover, in 35 patients, we observed the uptake of lipiodol in the MPVTT after TACE, and with the passage of time, collateral circulation was gradually established, and the incidences of bleeding and ascites

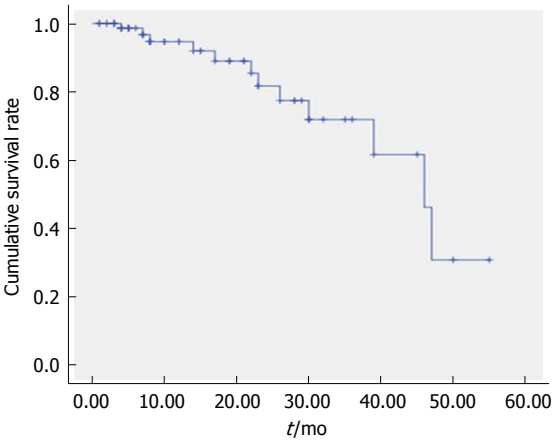

Figure 1 Kaplan-Meier curves of the overall survival for all 86 patients (months).

decreased. These changes may have contributed to the OS benefit (Table 1).

Thirty-five cases died within the first 6 mo, and these deaths were mainly due to bleeding and acute liver failure. Subsequently, the rate of death slowed (Figure 1).

Case 1

In a 47-year-old man with a HCC and MPVTT, an angiography *via* the proper hepatic artery revealed a tumor stain in the liver (Figure 2A), a stain in the right branch, and a portal trunk tumor thrombus (Figure 2B). We performed super-selective catheterization and administered embolization and chemotherapy infusion (Figure 2C). A post-operative CT scan revealed scattered deposition of lipiodol inside the MPVTT as well as intra-hepatic lesions (Figure 2D).

Case 2

In a 51-year-old woman with a HCC and MPVTT, a pre-operative CT scan revealed a tumor thrombus invading into the right branch and portal trunk (Figure 3A and B). Angiography *via* the celiac hepatic artery revealed a tumor stain in the liver and right branch and a portal trunk tumor thrombus (Figure 3C). A post-operative CT scan revealed the deposition of lipiodol inside the MPVTT (Figure 3D).

Complications

The main complications included acute variceal hemorrhaging, intractable ascites, and hepatic encephalopathy and failure. Twenty-eight cases exhibited variceal hemorrhaging after TACE; 19 of these complications occurred within 3 mo, and 14 of these patients died. Fourteen cases had intractable ascites after TACE, and, among the 15 cases with ascites before TACE, the ascites decreased or disappeared after the TACE.

Although a majority of the cases had background cirrhosis, acute liver failure was rare and was observed in six cases. Twenty-eight cases had moderately increased ALT and AST levels, but the T-Bil levels were slightly increased. Eight of these patients exhibited transient abnormal renal function, but function returned to normal within 1 wk in all cases. This phenomenon might have been due to the massive necrosis of the tumor cells.

Postembolization syndrome, which included pain and fever, occurred frequently and was successfully treated with conservative treatment. Twenty-five cases experienced nausea and loss of appetite. However, no cases had severe (grades 3 or 4) adverse effects, and symptoms were controllable with medical treatment. Myelosuppression was very rare; only eight cases exhibited slight reductions of the WBC and PLT count and recovered within 1 week without medical treatment.

DISCUSSION

The western guidelines of the Barcelona Clinic Liver Cancer (BCLC) staging system are frequently applied for decisions regarding initial treatments[14] for PVTT, but the management options for HCC with PVTT are more limited. To date, radiation and systematic chemotherapy or sorafenib targeted therapy[15] have been used for PVTT. However, the median survival time with sorafenib is only 6.5 mo in Asia[16]. In China, economic conditions restrict the application of sorafenib for most patients. Therefore, consecutive TACE is still used to treat selected patients with PVTT.

TACE has traditionally been considered to be contraindicated in cases of PVTT due to its high embolic effect and the potential for inducing hepatic necrosis and worsening liver dysfunction[6,16]. However, with progress of the technique, the effects of TACE have been confirmed in some randomized controlled trials (RCTs)[17]. A meta-analysis of prospective randomized trials demonstrated that survival is improved and good liver function is preserved after TACE for unresectable HCC[15] TACE can be safely performed in patients with HCCs that invade the main portal vein and may improve overall survival[18]. A meta-analysis of prospective randomized trials[19] demonstrated that TACE is potentially suitable and safe for patients with advanced HCC with PVTT, including patients with MPV obstructions. For selected patients with MPV obstructions, especially those with established collateral circulation and good liver function preservation, TACE treatment may prolong survival[20]. Although PVTTs are located outside of the liver parenchyma, their feeding artery still arises from the hepatic artery branches. We successfully embolized lesions in the liver and PVTTs *via* the hepatic artery branches in 86 cases; in the remaining eight patients, we still performed infusion chemotherapy *via* the hepatic artery despite the presence of shunts because we found stains of intra-hepatic lesions.

Hepatic arterial infusion chemotherapy has been investigated for the treatment of advanced HCC with portal vein tumor thrombosis in Asian countries. In

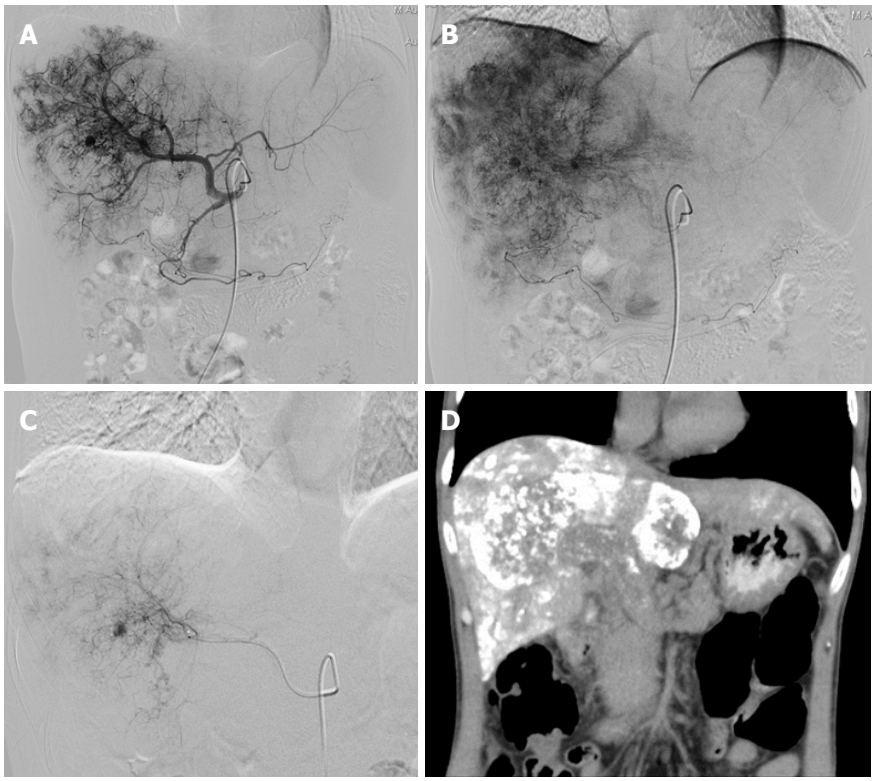

Figure 2 Images of case 1. A: Angiography *via* the proper hepatic artery revealed a tumor stain in the liver; B: Portal trunk tumor thrombus; C: Super-selective catheterization and administered embolization and chemotherapy infusion; D: Post-operative computed tomography (CT) scan revealed scattered deposition of lipiodol inside the major portal vein tumor thrombosis as well as intra-hepatic lesions.

this protocol, chemotherapeutic agent is infused into the hepatic artery *via* an implanted catheter, which reduces systemic side effects by first pass effects and maximizes drug delivery to the tumor. However, for HCC with MPVTT, patients always exhibit accompanying hypertension and hypersplenism. Thus, regular-dose chemotherapy may elicit more serious systemic toxicity, and patients may be more prone to have serious myelosuppression and gastrointestinal reaction and bleeding. Additionally, the nutrition conditions of MPTVV patients are generally poor, which makes a loss of appetite even more dangerous. Therefore, we need a more feasible protocol to ensure effectiveness while limiting the toxicity of chemotherapy.

In interventional therapy, the most feasible methods for reducing systemic toxicity are to reduce the dose or prolong the infusion time. However, doctors may worry that low doses could reduce the effect and wonder how a suitable dose and infusion time can be selected to ensure the effect. Where is the balance point? To find the answer, we must first understand the local plasma concentrations during the infusion process under different doses and whether these concentrations could meet our needs; subsequently, we can make the right decisions.

We performed a pharmacokinetics study with 1 mg/4 mg raltitrexed and 35 mg/150 mg oxaliplatin in a swine model. During infusion, both oxaliplatin and raltitrexed maintained high concentrations in the lobar hepatic artery during the whole infusion process. The highest concentrations of 35 mg and 150 mg of oxaliplatin at 4 h infusion time were 4259 μg/L and 14287 μg/L, respectively. The highest concentrations of 1 mg raltitrexed at 30 min, 60 min, and 120 min infusion times were 405, 212, and 128 μg/L, respectively. The corresponding highest concentrations in the 4-mg raltitrexed group were 1601, 902, and 587 μg/L. These results indicated that, even with the lowest dose and the longest infusion time, relatively high concentrations could be maintained.

However, we still needed to demonstrate how such high concentrations would act on tumor cells. We used the concentrations obtained from the pharmacokinetics study to perform an *in vitro* pharmacodynamics study in multiple tumor cell models. A MTT assay was used to measure the effects of oxaliplatin and raltitrexed on cell viability using different combinations of concentrations and times. We found that oxaliplatin and raltitrexed increased cell death in a concentration and time dependent manner. For oxaliplatin, a concentration of 5000-10000 μg/L and an infusion time of 4 h was a suitable combination. For raltitrexed, a suitable time was 60-120 min, and a suitable concentration was 250-500 μg/L.

By using super-selective or super super-selective techniques, we advanced a catheter into the segmental

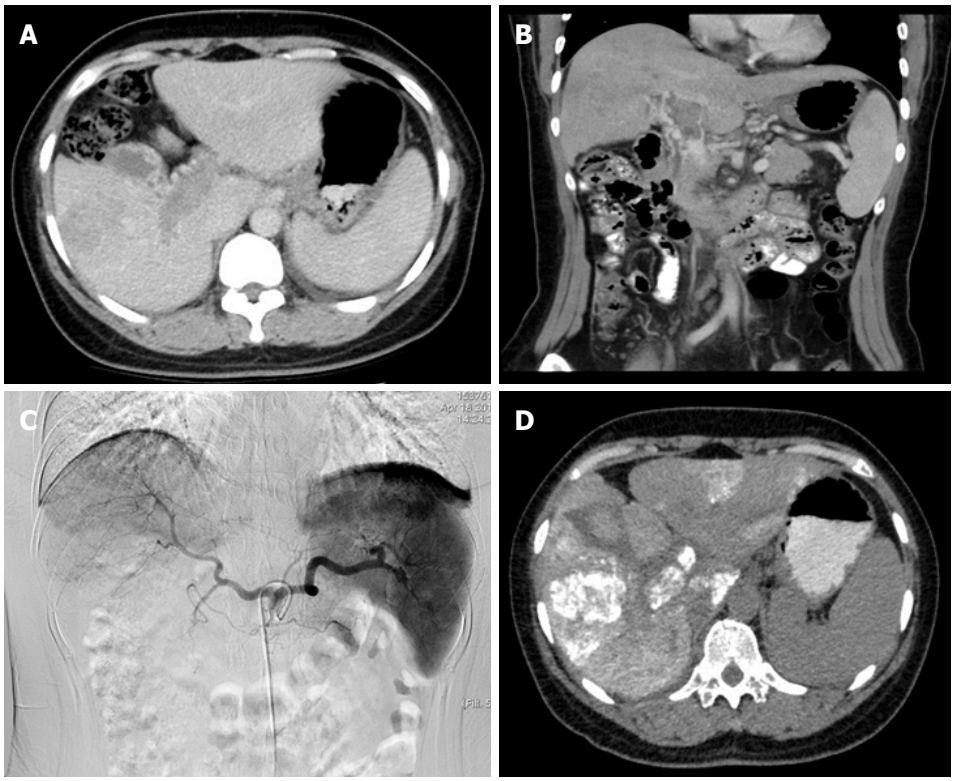

Figure 3 Images of case 2. A: Pre-operative CT scan (right branch); B: Pre-operative CT scan (portal trunk); C: Tumor stain in the liver and right branch and a portal trunk tumor thrombus; D: Post-operative CT scan revealed the deposition of lipiodol inside the major portal vein tumor thrombosis.

branch in most cases, and the blood flow in the target artery ranged from 1-2 mL/s. From this information, we could calculate the dose needed during infusion. For example, if we wanted to achieve a final 5000 μg/L concentration in the target artery, the required oxaliplatin could be calculated as 5000 (μg/L) × 14400 s × 1 (mL/s)/10^6 = 72 mg; if we wanted to achieve a final concentration of 500 μg/L in the target artery, the required raltitrexed could be calculated as 500 (μg/L) × 3600 s × 1 (mL/s)/10^6 = 1.8 mg.

Based on the results from these studies, 50 mg oxaliplatin was infused for 4 h, and 2 mg raltitrexed was infused for the next 1 h. This protocol produced good results and fewer complications. The 1-, 2-, and 3-year overall survival rates were 40.7%, 22.1%, and 8.1%, respectively. The complications were acceptable. Myelosuppression was observed in only eight patients. Twenty-eight patients exhibited bleeding, but 19 of these complications occurred within 3 mo after the TACE and may have been due to the establishment of collateral circulation and a decrease of portal vein pressure. Obviously, low-dose continuous hepatic arterial infusion chemotherapy can ensure a sufficient local concentration and an increase in cell death in tumor tissue, and longer infusion times mean that the tumor tissue can take up more of the agent. Additionally, the human body has more time to eliminate the agent, which dramatically reduces the toxicity.

The limitations of this study were the small number of patients and the retrospective design. Multivariate analysis may not precisely identify the factors associated with OS and PFS in such a small study.

In conclusion, this pharmacokinetic and pharmacodynamic study provided important information regarding the characteristics of drugs during continuous hepatic arterial infusion. TACE with low dose oxaliplatin and raltitrexed was effective and less toxic in patients with advanced HCC with MPVTT, making it a viable option for such patients.

ARTICLE HIGHLIGHTS

Research background

We had generated a pharmacokinetics and pharmacodynamics model for transarterial infusion. Here, we aimed to translate these findings into a clinical protocol for hepatocellular carcinoma (HCC) with portal vein tumor thrombus (PVTT).

Research motivation

Although transarterial chemoembolization (TACE) is widely used in the treatment of HCC with PVTT, the incidence of complication is high and overall survival is short, thereby limiting its use. More safe and effective protocols need to be developed to improve therapeutic effects.

Research objectives

We want to provide a simple and effect way to determine a clinical protocol based on pharmacokinetic and pharmacodynamic studies.

Research methods

After embolization, we kept the catheter in the feeding artery of the tumor and infused chemotherapy: oxaliplatin 50 mg in 250 mL of glucose was infused by pump for 4 h and raltitrexed 2 mg in 100 mL of 0.9% saline for the next 1 h after.

Research results

All cases received low dose continuous hepatic arterial infusion chemotherapy without major complications. Complete responses, partial responses, stable disease, and disease progression for intrahepatic disease were observed in 0, 45, 20, and 21 patient, respectively. The 1-, 2-, and 3-year overall survival rates of the 86 patients were 40.7%, 22.1%, and 8.1% respectively, and the median survival time was 8.7 mo.

Research conclusions

TACE with low dose continuous hepatic arterial infusion of oxaliplatin and raltitrexed could be safely used in major portal vein tumor thrombosis (MPVTT) patients. It is effective in patients with advanced HCC with MPVTT and is less toxic.

Research perspectives

Continuous hepatic arterial infusion chemotherapy was shown to be effective with limited complications. Based on results from pharmacokinetic and pharmacodynamics studies; we were able to choose agents and adjust the protocol with high efficiency.

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ORIGINAL ARTICLE

Clinical Trials Study

Clinical and prognostic significance of Raf kinase inhibitory protein expression in gastrointestinal stromal tumors

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Author contributions: Wang Y designed research; Wang Y performed research; Wang Y and Chen JJ contributed new reagents or analytic tools; Wang Y and Wang XF analyzed data; Wang Y wrote the paper; Wang Q edited the paper and provided primary revised opinion.

Institutional review board statement: The study was reviewed and approved by Sheng-Jing Hospital of China Medical University Institutional Review Board.

Informed consent statement: The patient involved in this study gave her written informed consent authorizing use and disclosure of her protected health information.

Conflict-of-interest statement: All the authors have no conflicts of interests to declare.

CONSORT 2010 statement: the guidelines of the CONSORT 2010 Statement have been adopted.

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Abstract

AIM

To detect the expression of Raf kinase inhibitory protein (RKIP) in gastrointestinal stromal tumors (GISTs) and to analyze its relationship with clinicopatholgical characteristics and prognosis of this disease.

METHODS

Sixty-three patients with pathologically diagnosed GISTs who underwent surgical resection at the Shengjing Hospital of China Medical University from January 2011 to January 2015 and had complete clinical, pathological, and follow-up data were included. Immunohistochemical method was used to detect the expression of RKIP in GIST tissue samples from these patients. Kaplan-Meier method was used to calculate the survival rate of 60 patients with complete follow-up data, and Cox regression analysis was performed to identify factors affecting the prognosis of patients GISTs to evaluate further the diagnostic and prognostic value of RKIP in GISTs.

RESULTS

In GIST tissues, RKIP positive signals, manifesting as brownish yellow or brown granules, were located in the cytoplasm or on the membrane. Of 63 tissue samples

included in this study, 34 (54%) were positive and 29 (46%) were negative for RKIP expression. Statistical analysis showed that RKIP expression in GISTs was significantly associated with tumor size, National Institutes of Health (NIH) risk grade, and mucosal invasion, but had no significant association with age, gender, tumor location, or the number of mitotic figures. Univariate Kaplan-Meier analysis revealed that the 1-, 3-, and 5-year survival rates were 94.4%, 89.2%, and 80.5% for patients with positive RKIP expression, and 88.6%, 68.2%, and 48.2% for patients with negative RKIP expression, suggesting that patients with high RKIP expression had significantly higher survival rates than those with low expression (Log-rank test, P = 0.0015). Cox regression analysis demonstrated that NIH risk grade was significantly associated with the prognosis of GISTs (P = 0.037), suggesting that NIH risk grade is a significant predictor of the prognosis of GISTs. RKIP expression had a tendency to predict the survival of GISTs (P = 0.122), suggesting that RKIP expression may have appreciated value to predict the prognosis of GISTs.

CONCLUSION

This study demonstrated that: (1) RKIP expression in GISTs is associated with tumor size, NIH risk grade, and mucosal invasion, and low or no expression of RKIP predicts a high malignancy potential; (2) high RKIP correlates positively with the survival of patients with GISTs; and (3) RKIP expression has appreciated value for predicting the survival of patients with GISTs, although it is not an independent prognostic factor in GISTs.

Key words: Gastrointestinal stromal tumors; Raf kinase inhibitory protein; Immunohistochemistry; Survival analysis

Core tip: In this study, the expression of Raf kinase inhibitory protein (RKIP) in gastrointestinal stromal tumors (GISTs) was examined by immunohistochemistry. We explored the relationship between RKIP protein expression and survival and prognosis in a large sample of GIST patients in China. RKIP protein expression was correlated with tumor growth, differentiation, malignancy, and the prognosis of the tumor. Our findings provide evidence for the diagnosis and prognosis assessment of GIST and offer new tumor treatment targets in GIST.

Wang Y, Chen JJ, Wang XF, Wang Q. Clinical and prognostic significance of Raf kinase inhibitory protein expression in gastrointestinal stromal tumors. *World J Gastroenterol* 2018; 24(23): 2508-2517 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2508.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2508

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) have previously been classified or pathologically diagnosed as leiomyosarcomas, leiomyomas, or leiomyoblastomas. For many years, surgical resection was the only effective treatment for GISTs. Until 1983, Mazur and Clark, two professors of pathology at the State University of New York, found that these "leiomyomas" have neither smooth muscle characteristics nor Schwann characteristics, and they for the first time proposed the concept of GISTs[1,2]. In 1998, Hirota *et al*[3] at the Osaka University reported that GISTs contained activated *c-kit* mutations. Further immunohistochemical examination showed that GIST tissue was positive for CD34 and CD117. Since then, the diagnosis of GISTs entered the standardization phase[4].

GISTs are now considered the most common gastrointestinal mesenchymal tumors, accounting for about 1%-4% of all gastrointestinal neoplasms. With the application of endoscopic ultrasound (EUS) in the gastrointestinal tract, more GISTs have been detected and distinguished form other subepithelial lesions[5-7]. The annual incidence of GISTs is about 2/100000[8,9]. Approximately 90% of GISTs are located in the stomach and small intestine, with gastric lesions being the most common (~60%). Geneticists estimate that about 10% of all individuals suffer from deleterious gene mutations[10], and current research shows that the development of GISTs is associated with multiple gene mutations, such as *c-kit* and *PDGFR*α (platelet derived growth factor receptor alpha) mutations. *C-kit* gene mutations generally occur in exon 11 (~60%) and include deletions, point mutations, and insertions. In some GISTs without *c-kit* mutations, *PDGFR*α mutations may occur, which account for about 8% of all GISTs. *PDGFR*α mutations occur mainly in exon 18 (~6% of all GISTs) and occasionally in exons 12 and 14 (~1.5% and 0.5% of all GISTs, respectively). Exons 18 and 12 encode the intracellular tyrosine kinase domain and jaxtamembrane domain, respectively. Of note, a small portion of GISTs are wild-type tumors, without *c-kit* or *PDGFR*α abnormalities.

GISTs are basically a malignancy, and there are almost no absolutely benign GISTs. Seemingly benign GISTs have the potential to be malignant. For many years, treatment of GISTs was limited to surgical resection and the application of chemotherapy regimens for sarcomas, but with poor efficacy. In 2000, the first successful case using the targeted drug "imatinib" in GISTs was reported. Imatinib is a small molecule tyrosine kinase inhibitor that targets c-kit and PDGFRα and can prevent the initiation of the downstream oncogenes by inhibiting theses tyrosine kinases.

The application of the targeted drug imatinib in GISTs was a milestone in the understanding and gene therapy of GISTs. However, with the increased

application of imatinib in clinical cases, drug resistance is beginning to emerge. Thus, it is important to investigate further the pathogenesis of GISTs and discover new therapeutic targets for this malignancy.

Raf kinase inhibitory protein (RKIP), also known as phosphatidylethanolamine-binding protein 1 (PEBP1), is an important endogenous modulator of many kinases and the star protein of recent oncology research. This protein was initially identified in the brain of cattle[11]. RKIP belongs to a highly conserved family of phospholipid-binding proteins, which contains more than 400 members and is widely distributed in microbes, plants, and mammals. RKIP can regulate multiple signaling transduction pathways, including the Raf/MAP kinase (MAPK) pathway, the β-adrenergic (β-AR) pathway, and the NF-kappa B pathway[12-14]. RKIP is not only a kinase inhibitor but also a target of phosphorylation, having important and complex functions, such as regulating tumor growth and metastasis and affecting cell cycle and apoptosis.

Recently, there have been many studies on RKIP, including in gastric cancer, colon cancer, esophageal cancer, and gynecological tumors. However, there have been very few reports on RKIP in GISTs. The role of this protein in the growth and metastasis of GISTs, the relationship between RKIP expression and clinical characteristics of GISTs, and the effect of RKIP expression on the prognosis of GISTs remain unclear. To address these problems, we detected the expression of RKIP in GISTs by immunohistochemistry and analyzed the clinical and prognostic significance of RKIP expression in GISTs, with an aim to provide a basis for GIST diagnosis, prognosis evaluation, and identification of new tumor therapeutic targets.

MATERIALS AND METHODS

Patients

Sixty-three patients with pathologically diagnosed GISTs who underwent surgical resection at the Shengjing Hospital of China Medical University from January 2011 to January 2015 and had complete clinical, pathological, and follow-up data were included in this study. There were 33 men and 30 women, with a mean age of 56.2 years (range, 21-83 years). The location of GISTs included the stomach (n = 35, 55.6%), duodenum (n = 12, 19.0%), jejunoileum (n = 14, 22.2%), and colon (n = 2, 3.1%).

The diagnostic criteria for GISTs were histopathological features consistent with GISTs and immunohistochemical positivity for CD117, immunohistochemical negativity for CD117 but positivity for CD34, or immunohistochemical negativity for CD117 and CD34 as well as smooth muscle actin (SMA), desmin, and S-100 (to exclude smooth muscle tumors and neurogenic tumors). There were 59 (90.8%) cases positive for CD117 and 50 (76.9%) cases positive for CD34 (Table 1).

GISTs were graded based on the National Institutes of Health (NIH) consensus on defining the risk of aggressive behavior: (1) Very low risk of aggressive behavior (grade I), tumor size < 2 cm and mitotic figures < 5/50 high-power fields (HPFs); low risk (grade II), tumor size of 2-5 cm and mitotic figures < 5/50 HPFs; moderate risk (grade III), tumor size of 5-10 cm and mitotic figures < 5/50 HPFs, or tumor size < 5 cm and mitotic figures of 6-10/50 HPFs; and high risk (grade IV), tumor size > 5 cm and mitotic figures > 5/50 HPFs, or tumor size > 10 cm and mitotic figures > 10/50 HPFs.

Table 1 Clinicopathological data of the 63 patients with gastrointestinal stromal tumors

Characteristic	Patients (n = 63)	
	Number	Percentage (%)
Gender		
Male	33	52.4
Female	30	47.6
Age (yr)		
≥ 56	32	50.8
< 56	31	49.2
Tumor location		
Stomach	35	55.6
Duodenum	12	19.0
Jejunoileum	14	22.2
Colon	2	3.2
Tumor size (cm)		
< 2	11	17.5
2-5.9	20	31.7
6-10	18	28.6
> 10	14	22.2
NIH risk grade		
Very low	10	15.9
Low	19	30.1
Moderate	10	15.9
High	24	38.1
Mitotic figures per 50 HPFs		
0	11	17.5
1-4	35	55.6
5-9	6	9.4
>10	11	17.5
Mucosal invasion		
Yes	33	52.4
No	30	47.6

HPFs: High-power fields.

Of 63 cases of GISTs included in this study, three died from other reasons and the remaining 60 had complete follow-up data, with a mean follow-up period of 48 mo (range, 5-61 mo). Tumor-adjacent tissue samples were collected as controls.

Immunohistochemical staining

GIST tissue samples were fixed in formalin, embedded in paraffin, and cut into 4 μm sections. The sections were then routinely dewaxed and rehydrated. After endogenous peroxidase was inactivated with H_2O_2, heat-mediated antigen retrieval was performed. The sections were then blocked with 5% bovine serum albumin (BSA) blocking solution for 20 min at room temperature. Subsequently, the sections were incubated with primary

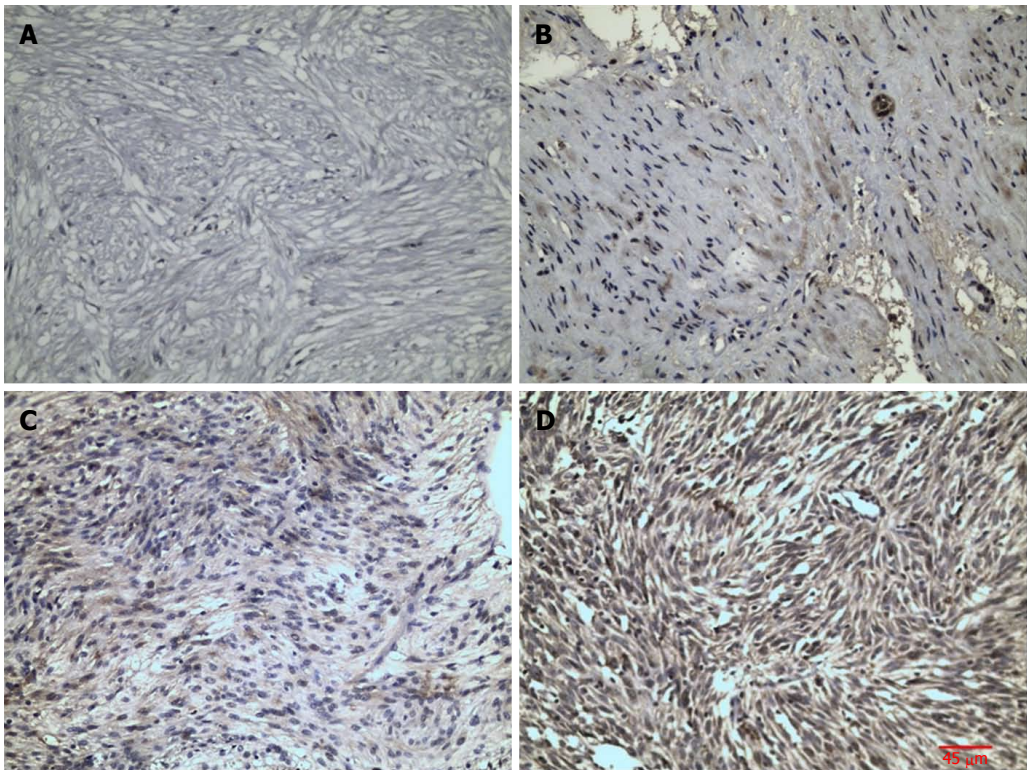

Figure 1 Immunohistochemcial staining for Raf kinase inhibitory protein in gastrointestinal stromal tumor tissues. A: Negative expression; B: Mildly positive expression; C: Moderately positive expression; D: Strongly positive expression. Scale bars on Figure. Magnification is 200 ×.

antibody (rabbit anti-RKIP antibody) for 12 h at room temperature, followed by incubation with secondary antibody (biotinylated goat anti-rabbit IgG) for 20 min at 25 ℃. After that, the sections were incubated with SABC-AP/BCIP reagents to develop the color. Finally, the sections were counterstained with nuclear fast red, dehydrated, mounted, and observed under a microscope.

Evaluation of immunohistochemical staining

Positive immunohistochemical staining signals for RKIP were present in the cytoplasm or on the membrane. A semi-quantitative method that combines staining intensity and the percentage of positive cells was adopted to evaluate the expression of RKIP. Ten HPFs were randomly selected from a slide, and 100 cells were counted in each HPF to calculate the percentage of positive cells. The staining intensity was graded as follows: 0, no staining; 1, yellow; 2, brownish yellow; and 3, brown. The percentage of positive cells was scored as follows: 0, < 25%; 1, 25% to 50%; 2, 51% to 75%; and 3, > 75%. The overall score was the product of the staining intensity and the percentage of positive cells and graded as negative (0-2), mildly positive (+, 3), moderately positive (++, 4-6), or strongly positive (+++, 9). RKIP expression was judged to be either negative (0-2) or positive (3-9).

Follow-up

The patients were followed by telephone, outpatient visits, or letters. Survival time was defined as the period from the date of surgery to the date of the last follow-up or death. Of the 63 cases included, 60 had complete follow-up data and three died of other diseases or accidents. The follow-up period was between January 2013 and December 31, 2017.

Statistical analysis

SPSS17.0 software was used for all statistical analyses. Percentages were compared using the chi-square test. Survival curves were plotted with GRAPHPAD software, and survival analysis was performed using the Kaplan-Meier method and the log-rank test. Multivariate prognostic analysis was performed using a Cox regression model. *P*-values < 0.05 were considered statistically significant.

RESULTS

Relationship between RKIP expression and clinicopathological characteristics of GISTs

In GIST tissue, RKIP positive signals, manifesting as brownish yellow or brown granules, were located in the cytoplasm or on the membrane (Figure 1). Of 63 tissue samples included in this study, 14 were strongly positive (+++), 16 moderately positive (++), four mildly positive (+), and 29 negative for RKIP expression (Figure 1A-D, respectively). In total, 34 (54%) cases were positive and 29 (46%) were negative for RKIP expression. Statistical analysis showed that RKIP expression in GISTs was significantly associated with

Table 2 Relationship between raf kinase inhibitory protein expression and clinical and pathological characteristics of gastrointestinal stromal tumors

Characteristic	Number	RKIP expression		*P* value
		Positive (≥ 3)	Negative (< 3)	
Gender				
Male	33	19	14	0.547
Female	30	15	15	
Age (yr)				
≥ 56	32	20	12	0.167
< 56	31	14	17	
Tumor location				
Stomach	35	16	19	0.503
Duodenum	12	8	4	
Jejunoileum	14	9	5	
Colon	2	1	1	
Tumor size (cm)				
< 2	11	10	1	< 0.01[1]
2-5	20	15	5	
6-10	18	5	13	
> 10	14	4	10	
NIH risk grade				
Very low	10	8	2	< 0.01[1]
Low	19	16	3	
Moderate	10	3	7	
High	24	7	17	
Mitotic figures per 50 HPFs				
0	11	8	3	0.218
1-4	35	20	15	
5-9	6	2	4	
> 10	11	4	7	
Mucosal invasion				
Yes	33	13	20	0.015[1]
No	30	21	9	

[1]RKIP positive percentage were compared using the χ test, $P < 0.05$ were considered statistically significant. RKIP: Raf kinase inhibitory protein; HPFs: High-power fields.

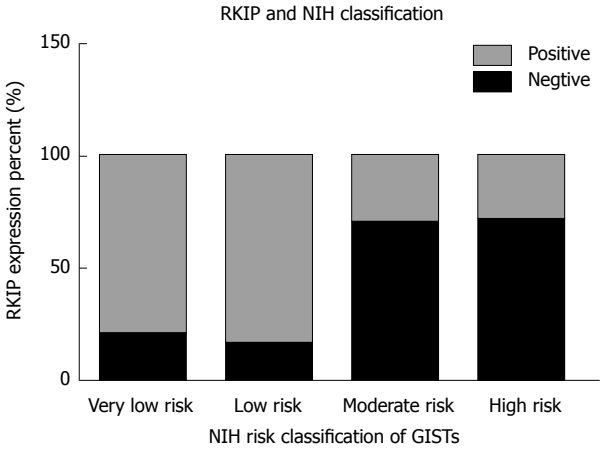

Figure 2 Relationship between Raf kinase inhibitory protein expression and National Institutes of Health risk grade in gastrointestinal stromal tumors. The figure shows RKIP positive rate is different between each NIH risk grade group: The higher risk grade group with the lower RKIP positive rate. RKIP: Raf kinase inhibitory protein; NIH: National Institutes of Health; GISTs: Gastrointestinal stromal tumors.

tumor size, NIH risk grade, and mucosal invasion. The positive rates of RKIP expression were 80.0%, 84.2%, 30.0%, and 29.2% in the very low risk, low risk, moderate risk, and high risk groups, respectively ($P < 0.01$); 90.9%, 75.0%, 27.8%, and 28.6% in tumors of < 2 cm, 2-5.9 cm, 6-10 cm, and > 10 cm, respectively ($P < 0.01$); and 39.4% and 70.0% in tumors with and without mucosal invasion, respectively ($P = 0.015$). However, RKIP expression had no significant association with age, gender, tumor location, or the number of mitotic figures (Table 2, Figures 2-4).

Follow-up data

Of 60 patients who were followed, two were lost to follow-up, and the rate of follow-up was 92.1%. Fourteen patients died of GISTs. The median survival time was 54.96 mo. The 1-, 3-, and 5-year survival rates for all patients were 91.7%, 83.3%, and 71.7%, respectively, and the mean survival time was 47.66 ± 16.61 mo (Figure 5).

Relationship between RKIP expression and prognosis of GISTs

Univariate Kaplan-Meier analysis revealed that the survival rates differed significantly between patients with positive and negative RKIP expression (Log-rank test, $P = 0.0015$) (Figure 6). The 1-, 3-, and 5-year survival rates were 94.4%, 89.2%, and 80.5% for patients with positive RKIP expression, and 88.6%,

Table 3 Multivariate analysis of factors affecting prognosis of gastrointestinal stromal tumors (n = 63)

	B	SE	Wald	df	significance	Exp (B)
NIH risk grade	1.299	0.624	4.337	1	0.037	3.664
Age	-0.008	0.019	0.186	1	0.667	0.992
Sex	-0.500	0.561	0.795	1	0.373	0.606
RKIP expression	1.049	0.678	2.395	1	0.122	2.855
Mitotic figures	0.087	0.283	0.095	1	0.758	1.091
Tumor size	-0.121	0.435	0.078	1	0.781	0.886
Tumor location	0.269	0.351	0.589	1	0.443	1.309

NIH: National Institutes of Health; RKIP: Raf kinase inhibitory protein.

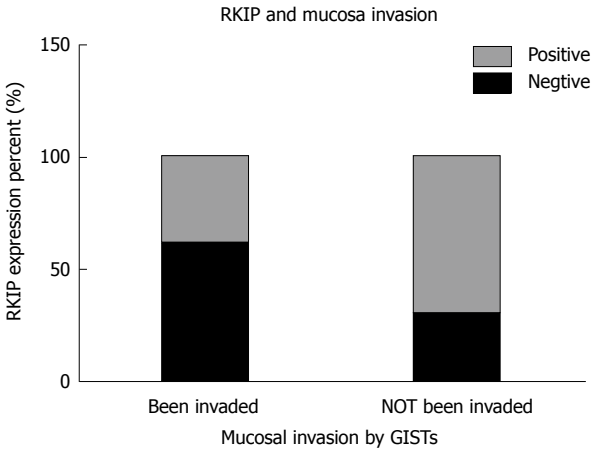

Figure 3 Relationship between Raf kinase inhibitory protein expression and mucosal invasion in gastrointestinal stromal tumors. RKIP expression is related with mucosal invasion status in GISTs: the RKIP positive rate of the mucosal invaded group is lower than the control group whose mucosa has not been invaded (P < 0.05). RKIP: Raf kinase inhibitory protein; GISTs: Gastrointestinal stromal tumors.

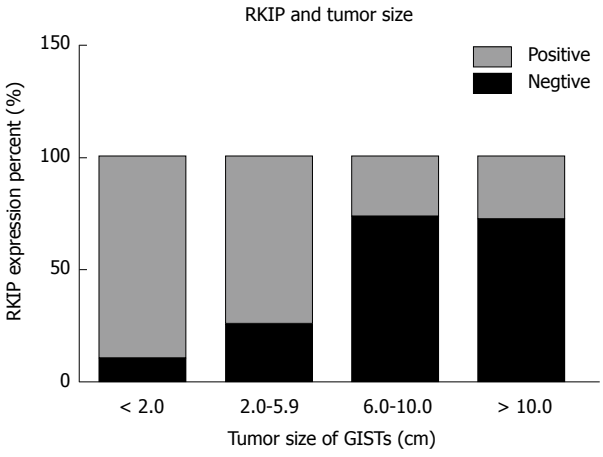

Figure 4 Relationship between Raf kinase inhibitory protein expression and tumor size in gastrointestinal stromal tumors. RKIP positive rate declined as the tumor size increased. RKIP: Raf kinase inhibitory protein; GISTs: Gastrointestinal stromal tumors.

68.2%, and 48.2% for patients with negative RKIP expression, suggesting that patients with high RKIP expression had higher survival rates than those with low expression.

Multivariate analysis of factors affecting prognosis of GISTs

Cox regression analysis demonstrated that NIH risk grade was significantly associated with the survival of GISTs (P = 0.037) (Table 3), suggesting that NIH risk grade is a significant predictor of the prognosis of GISTs. Of note, RKIP expression had a tendency to predict the survival of GISTs (P = 0.122), suggesting that RKIP expression may have appreciated value to predict the prognosis of GISTs. In contrast, other factors, including age, gender, number of mitotic figures, and tumor size, did not significantly predict the prognosis of GISTs (Table 3).

DISCUSSION

GISTs are the most common gastrointestinal mesenchymal neoplasms. In the past 30 years, the pathogenesis of GISTs has gradually been elucidated, and their diagnosis and treatment has become standardized[15-19]. However, there is still a significant number of recurrent or metastatic GISTs. Because of their resistance to radiation and chemotherapy, recurrent or metastatic GISTs were once considered an incurable disease. GIST patients with metastasis have a median survival period as low as 20 mo, and for patients with locally recurrent GISTs, the median survival period is only 9-12 mo[20,21]. Imatinib, which is a drug that targets *c-kit* and *PDGFR*α gene mutations, is the first choice of molecular therapy for GISTs. However, there are currently no other targets for therapy and prognosis monitoring in GISTs.

RKIP is a structurally complex protein that can act as a "multidirectional switch" to regulate multiple signaling transduction pathways, including the Raf/MAPK pathway, the β-AR pathway, and the NF-kappa B pathway[12,13]. RKIP protein is not only a kinase inhibitor but also a target of phosphorylation. For example, in the MAPK pathway, RKIP binds and inhibits Raf protein, and in the G protein-coupled pathway, RKIP binds to GRK2. This functional shift is achieved through the phosphorylation of the S153 residue by protein kinase C (PKC)[22]. Recently, the regulatory function of RKIP protein in tumors has gradually become a hot research topic, but the data on its expression in GISTs are limited.

In this study, we found in GIST tissue that RKIP

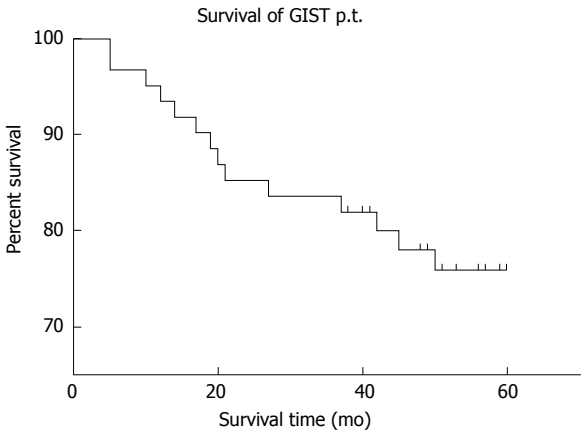

Figure 5 Survival rate of 60 gastrointestinal stromal tumors patients at follow-up. GIST: Gastrointestinal stromal tumors.

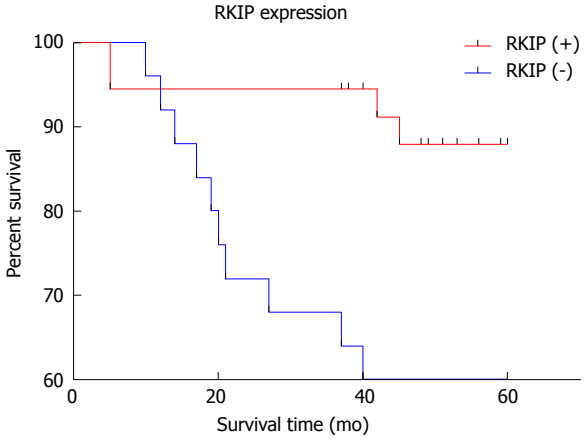

Figure 6 Relationship between raf kinase inhibitory protein expression and prognosis in gastrointestinal stromal tumors. Compared with the RKIP negative Group, the high RKIP expression group is correlated with a better survival rate, the difference was significant. (Log-Rank analysis, $P = 0.0015$). RKIP: Raf kinase inhibitory protein.

was mainly expressed in the cytoplasm and on the membrane, presenting as brownish yellow or brown granules. Such an expression pattern is consistent with many previous reports. RKIP is mainly expressed in the human cytoplasm or on the membrane. The *RKIP* gene is located on chromosome 12q24.23 and contains four exons, spanning a length of 1435 bp and encoding a 21 KDa protein containing 187 amino acid residues[23]. Immunohistochemical results showed that the expression of RKIP in GISTs tissue was correlated with tumor size; in tumors of < 2 cm, 3-5.9 cm, 6-10 cm, or > 10 cm, the positive expression rates of RKIP were 90.9%, 75%, 27.8%, and 28.6%, respectively ($P < 0.01$). As the tumor size increased, the degree of malignancy increased, and the expression of RKIP was gradually reduced or absent. This result suggests that the expression of RKIP may be associated with the growth of GISTs. Consistent with this finding, Eves *et al*[24] reported the lack of RKIP protein expression in hepatoma cells, which can lead to rapid cell division and increased cell proliferation instability[25]. As far as the mechanism is concerned, RKIP protein is an important regulatory factor in the MAPK signaling transduction pathway, and its ligand Raf-1 can regulate the cell cycle of mitotic cells[26]. In addition, the activation of downstream protein ERK1/2 in the MAPK pathway can control important structures that are related to cell division, such as centromeres, spindles, and intermediates[27,28]. Collectively, RKIP can regulate tumor cell cycle and mitosis by regulating the phosphorylation and activation of Raf-1 and the activation of downstream MEK/ERK. In GIST cells, the low or no expression of RKIP protein can reduce its inhibitory effect on the MAPK pathway, thus resulting in the proliferation of tumor cells[29]. Therefore, there is a negative correlation between tumor volume and RKIP expression.

When analyzing the relationship between RKIP protein expression and tumor invasion, we found that there was a significant difference in the positive rate of RKIP between GISTs with and without mucosal invasion ($P = 0.015$). Previously, many reports on RKIP in other tumor types have found that RKIP protein was related to invasion or metastasis suppression, suggesting a general role of RKIP protein in tumor metastasis and invasion. In the earliest research on RKIP in prostate cancer, Fu *et al*[30] performed a gene chip analysis of mRNA expression in a prostate cancer cell line with a low metastatic potential (LNCaP cells) and a prostate cancer cell line with a high metastatic potential (C42B cells) and found that the expression level of RKIP was lower in C42B cells than in LNCaP cells. The study of specimens from patients with prostate cancer also showed that the expression of RKIP was lower in metastatic prostate cancer. These results suggest that there is a correlation between RKIP expression and tumor metastasis. Furthermore, an *in vitro* tumor invasion assay demonstrated that down-regulation of RKIP expression in LNCaP cells can enhance their invasion ability, while restoring RKIP expression in C42B cells can weaken their ability of invasion[31]. In both *in vivo* and *in vitro* studies of prostate cancer and melanoma, high expression of RKIP can reduce vessel invasion and reduce metastasis risk[32]. Similar results have also been reported in studies on breast cancer with lymph node metastasis[33], insulinoma[34], colon cancer[35,36], liver cancer[25], ovarian cancer[37], and thyroid cancer[38].

In the present study, the positive rate of RKIP expression differed significantly among different NIH risk grades (very low risk: 80.0%; low risk: 84.2%; moderate risk: 30.0%; high risk: 29.2%; $P < 0.01$). Fletcher discovered that tumor size and the number of mitotic figures are the main prognostic factors to evaluate the malignant potential of GISTs; and, therefore, proposed a risk grading system (NIH grading), which is of great importance in clinical and pathological diagnosis. In this study, the expression of RKIP was correlated with the size of GISTs: the larger the tumor and the higher the malignancy, the higher the possibility of negative expression of RKIP. However, our results indicated that

the expression of RKIP did not significantly correlate with the number of mitotic figures. This result is similar to the study of Miettinen, who found that small intestinal stromal tumors had higher malignant potential than gastric stromal tumors. Gastric stromal tumors had good biological behavior as long as the number of mitotic figures was no more than 5/50 HPFs, and duodenal stromal tumors had good prognosis only when the number of mitotic figures was less than 2/50 HPFs[39]. These findings suggest that other factors (such as tumor location) may also affect the weight of the number of mitotic figures as one of the only two indicators of the NIH risk grading system. To overcome this problem, Miettinen further proposed a new risk assessment system to use anatomical site as an independent assessment factor. In 2013, Sebatian *et al*[40] performed an immunohistochemical analysis of 161 surgical specimens and a survival analysis based on the clinical and pathological data of these surgical patients and found that the low expression of RKIP was significantly correlated with both high NIH risk grade (P = 0.033, moderate *vs* low risk group) and high Miettnen grade (P = 0.044, moderate *vs* low risk group).

In order to have a more direct understanding of the impact of RKIP expression on the prognosis of patients with GISTs, we followed 60 GISTs patients (median follow-up period, 54.96 mo) and plotted the survival curve of these patients according to RKIP expression using univariate Kaplan-Meier method. The results showed that the survival rate differed significantly between patients with positive and negative expression of RKIP (Log-rank test, P = 0.0015). The 1-, 3-, and 5-year survival rates were 94.4%, 89.2%, and 80.5% for patients with positive RKIP expression, and 88.6%, 68.2%, and 48.2% for patients with negative RKIP expression, suggesting that patients with high RKIP expression had higher survival rates than those with low expression. Thus, RKIP expression has appreciated value for evaluating the prognosis of patients with GISTs.

Further, Cox regression analysis showed that the prognosis of GISTs patients was related to NIH risk grade (P = 0.037), indicating that NIH risk grade is an important parameter to evaluate the prognosis of patients. RKIP expression had a tendency to predict the prognosis of GISTs (P = 0.122), suggesting that RKIP expression may have appreciated value to predict the survival of GISTs. RKIP as a prognostic factor has been reported in many other tumors. For example, Fu *et al*[41] used prostate cancer tissue chip to detect RKIP expression in non-tumor tissues, primary tumor tissues, and metastasis tumor tissues and found that RKIP was strongly expressed in the majority of non-tumor tissues. Although RKIP was weakly expressed in advanced prostate cancer (Gleason score, 6-7), it was strongly expressed in the rest prostate cancer tissues. RKIP expression, however, almost disappeared in metastasis tumor tissues. These results are consistent with the above-mentioned observation that RKIP may act as a metastasis suppressor gene, and also indicate that RKIP can be used as a prognostic factor for prostate cancer. In gliomas, the absence of RKIP correlated with a higher malignancy and a shorter survival period. Yu *et al*[42] performed a meta-analysis of RKIP expression in gastrointestinal tumors, in which they systematically summarized and analyzed 28 articles that met the criteria, and found that low expression of RKIP is associated with a poor prognosis and short survival time.

Although both univariate and multivariate analyses indicated that RKIP expression had appreciated significance in prognosis evaluation in patients with GISTs in this study, multivariate analysis demonstrated that RKIP expression cannot be an independent prognostic factor in GISTs (P = 0.122). Similarly, Marcus Valadao concluded that RKIP expression does not affect the overall survival (P = 0.73), progression free survival (P = 0.22), or objective response rate (P = 0.30)[43]. Sebatian *et al*[40] performed a multiple regression analysis of factors affecting overall survival (age, gender, imatinib chemotherapy, Flecher grade, *etc*.) in patients with GISTs and suggested that low expression of RKIP does not have prognostic significance. This may be because current studies are all limited to detection of RKIP expression at the protein level using immunohistochemical method. Because of the qualitative nature and limited number of experimental samples, there is a lack of quantitative research and detection of RKIP expression at the genetic level.

In conclusion, this study demonstrated that: (1) RKIP expression in GISTs is associated with tumor size, NIH risk grade, and mucosal invasion, and low or no expression of RKIP predicts a high malignancy potential; (2) high RKIP correlates positively with the survival of patients with GISTs; and (3) RKIP expression has appreciated value for predicting survival of patients with GISTs, although it is not an independent prognostic factor in GISTs. These findings suggest that RKIP expression in GISTs is closely related to the diagnosis and treatment of this disease and RKIP may be used as a novel target for therapy and prognostic evaluation in GISTs. Future studies should further investigate the role and underlying mechanism of RKIP in GISTs.

ARTICLE HIGHLIGHTS

Research background

Gastrointestinal stromal tumors (GISTs) are the most common gastrointestinal mesenchymal tumor. Raf kinase inhibitory protein (RKIP) protein is both a kinase inhibitor and a phosphorylation target. It has important and complex functions in regulating tumor growth, metastasis, cell cycle, and apoptosis. Many recent articles on RKIP proteins have reported a link to various cancers, including gastric cancer, bowel cancer, esophageal cancer, and gynecologic oncology. However, there are only a few references on the expression of RKIP protein in gastrointestinal stromal tumors.

Research motivation

The following problems, which we need to solve urgently, are also the research motivation of this article: (1) The function of the RKIP in the GISTs neoplastic

generation and metastasis; (2) the relationship between the RKIP expression and GIST clinical data; and (3) the relationship between the prognosis of GIST and RKIP.

Research objectives

In this study, we examined the expression of RKIP in GISTs by immunohistochemistry to explore the clinical significance of GIST expression and its prognosis. This research will provide evidence and support for the diagnosis of GIST, prognostic evaluation, and new tumor treatment targets

Research methods

The study included 63 cases of paraffin-embedded specimens of surgically resected and pathologically confirmed clinical specimens from Shengjing Hospital Affiliated to China Medical University. All the cases are clinically and pathologically complete, dated from January 2011 to January 2015. The expression of RKIP protein was analyzed by immunohistochemistry. The Kaplan-Meier method was used to calculate the overall survival of 60 patients followed up for survival analysis. The prognostic significance of each index was analyzed by COX multiple regression to clarify further the value of RKIP protein level in the diagnosis and prognosis of GISTs.

Research results

In GIST tissues, RKIP protein was positively expressed in the cytoplasm and cell membrane with brownish-yellow or brown granules. RKIP protein positive expression was found in 34 (54%) of the 63 specimens in this experiment, and the total negative expression was 29 (46%). The RKIP positive expression was related with GIST tumor size, NIH grade, and mucosal invasion. RKIP and age, gender, tumor location, and how many mitotic figures were not related. Kaplan-Meier method was used to draw the survival curves related to RKIP differential expression. The results showed that the 1, 3, and 5-year survival rates of RKIP positive group were 94.4%, 89.2% and 80.5%, respectively. The survival rates of RKIP negative group at 1, 3, and 5 years were only 88.6%, 68.2%, and 48.2%, respectively. Comparing with the RKIP negative Group, the RKIP high expression group was correlated with a better survival rate (Log-Rank analysis, $P = 0.0015$). The results of the COX multivariate analysis showed that the prognosis of patients with GISTs was related to NIH grade ($P = 0.037$) and Exp (B) was 3.664, indicating that NIH risk grade was an important factor to evaluate the prognosis of patients. However, the expression of RKIP correlated with the prognosis of patients ($P = 0.122$). The Exp (B) value was 2.855, suggesting that RKIP expression may have some reference value for the survival of GIST.

Research conclusions

The expression of RKIP protein in GISTs correlated with tumor size, NIH stage, and invasiveness of the mucosa (invasion degree), and each index suggested that the higher the degree of malignancy was associated with lower or more loss of RKIP expression. When compared with other factors (age, sex, tumor location, *etc.*), there was no relationship with RKIP level. RKIP overexpression was positively correlated with the survival of patients with GISTs, which has some implications for the prognosis of patients. RKIP expression has certain reference value for the survival of GIST, but it cannot be used as an independent factor to evaluate the prognosis of GISTs.

Research perspectives

As a complex protein, RKIP has a "multi-directional switch" function on many cell conduction pathways. RKIP remains a hot topic in recent cancer research. However, data on the relationship between RKIP and the pathogenesis and treatment of GIST still remains unclear. Additional studies on the mechanisms underlying RKIP expression disorder will possibly find that RKIP protein is marker protein for prognostication of GIST. In addition to its potential as a monitoring indicator, regulation and target treatment of RKIP could be a treatment option for GIST. Thus, RKIP may have practical value in understanding the biological characteristics and expression of GIST.

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META-ANALYSIS

Comparing outcomes for endoscopic submucosal dissection between Eastern and Western countries: A systematic review and meta-analysis

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Author contributions: Daoud DC designed the study, collected and analyzed the data and wrote the manuscript; Suter N collected and analyzed the data; Durand M performed the statistical analysis and analyzed the data; von Renteln D designed the study, analyzed the data and wrote the manuscript; Bouin M and Faulques B revised the manuscript critically for intellectual content; and all the authors provided intellectual input for the study and approved the final version of the manuscript.

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Abstract

AIM

To compare endoscopic submucosal dissection (ESD) outcomes between Eastern and Western countries.

METHODS

A systematic review and meta-analysis was performed using PubMed, MEDLINE, Web of Science, CINAHL and EBM reviews to identify studies published between 1990 and February 2016. The primary outcome was the efficacy of ESD based on information about either curative resection, *en bloc* or R0 resection rates. Secondary outcomes were complication rates, local recurrence rates and procedure times.

RESULTS

Overall, 238 publications including 84318 patients and 89512 gastrointestinal lesions resected using ESD were identified. 90% of the identified studies reporting ESD

on 87296 lesions were conducted in Eastern countries and 10% of the identified studies reporting ESD outcomes in 2216 lesions were from Western countries. Meta-analyses showed higher pooled percentage of curative, *en bloc*, and R0 resection in the Eastern studies; 82% (CI: 81%-84%), 95% (CI: 94%-96%) and 89% (CI: 88%-91%) compared to Western studies; 71% (CI: 61%-81%), 85% (CI: 81%-89%) and 74% (CI: 67%-81%) respectively. The percentage of perforation requiring surgery was significantly greater in the Western countries (0.53%; CI: 0.10-1.16) compared to Eastern countries (0.01%; CI: 0%-0.05%). ESD procedure times were longer in Western countries (110 min *vs* 77 min).

CONCLUSION

Eastern countries show better ESD outcomes compared to Western countries. Availability of local ESD expertise and regional outcomes should be considered for decision making to treat gastrointestinal lesions with ESD.

Key words: Curative resection; *En bloc* resection; R0 resection; Perforation

Core tip: Endoscopic submucosal dissection (ESD) has become the preferred approach to remove larger or advanced gastrointestinal lesions in Asian countries. However, there might be regional differences in outcomes since the majority of ESD publications come from the Eastern world. To provide such information we conducted a systematic review and meta-analysis comparing ESD outcomes for different regions of the world. This study found that there are indeed regional differences for ESD outcomes. Eastern countries had better curative, *en bloc* and R0 resection rate than Western countries as well as less perforation requiring surgery.

Daoud DC, Suter N, Durand M, Bouin M, Faulques B, von Renteln D. Comparing outcomes for endoscopic submucosal dissection between Eastern and Western countries: A systematic review and meta-analysis. *World J Gastroenterol* 2018; 24(23): 2518-2536 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i23/2518.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i23.2518

INTRODUCTION

Digestive cancers account for 20% of cancers diagnosed worldwide. The global age-standardized incidence rate (ASR) of esophageal, stomach and colorectal cancer are 5.9, 12.1 and 17.2 per 100000 respectively. Some geographic differences in the incidence of gastrointestinal cancers exist. Colorectal cancer rates are considerably higher in Western countries, while gastric cancer is the most prevalent gastrointestinal cancer in Asian countries. Esophageal squamous cell cancer is predominant in Eastern countries, but the incidence of esophageal adenocarcinoma has been increasing significantly in several Western countries[1]. Curative endoscopic therapy is possible for such gastrointestinal cancers or precancerous lesions if they are detected at a stage where the risk of lymph node metastasis is low[2].

Endoscopic submucosal dissection (ESD) has been developed in Japan to allow for endoscopic *en bloc* and curative removal of larger superficial gastrointestinal lesions and early gastrointestinal cancers according to oncologic standards (R0 and *en bloc* resection)[3,4]. It is assumed that *en bloc* resection translates into lower recurrence rates compared to other endoscopic treatment options such as endoscopic mucosal resection (EMR). EMR requires the removal of gastrointestinal lesions > 20 mm, usually in several pieces, thus making it impossible to confirm a complete resection in histopathology with margins free of dysplastic or cancer tissue[5,6]. Consequently, ESD has become the preferred approach to remove larger or advanced gastrointestinal lesions in many Asian countries. Furthermore, previous systematic reviews comparing ESD to EMR have come up with the conclusion that ESD yields better results for complete and *en bloc* removal compared to EMR[7-11].

Despite these results, widespread adoption of ESD has remained limited in Western countries and EMR continues to be the mainstay of endoscopic therapy. ESD is known to have high complication rates, demands long procedure times and requires substantial training and expertise development[7-11]. Differences in incidence (*e.g.,* for gastric cancer) provide different local exposure to develop adequate ESD skills. Furthermore, differences in remuneration systems (*e.g.,* fee per service systems without specific procedure codes) are other factors that might influence regional uptake of ESD. Such regional differences of case load and/or lesions suitable to develop ESD skills might translate into different regional outcomes for ESD. Consequently, Western centers might have different results and such differences are important to consider for clinical decision-making. Previous studies have focused on comparing ESD to EMR and without considering that there might be differences for regional ESD outcomes[7-11]. To provide such information we conducted a systematic review comparing ESD outcomes for different regions of the world.

MATERIALS AND METHODS

We conducted a systematic review of the literature and meta-analysis, and reported our results in accordance to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) recommendations.

Search strategy

A computerized, systematic bibliographic search was performed on PubMed, MEDLINE (OVID), EMBASE

(OVID), Web of Science (Clarivate Analytics), CINAHL (EBSCO) and EBM reviews (OVID) databases to identify relevant publications. The search keywords were "endoscopic submucosal dissection" and "ESD." The search was limited by the non-restrictive filters "Human", "Clinical Trial" and publication language "English". For each database, terms and expressions from controlled vocabulary (MeSH, EMTREE, *etc*.) were used. Free-text searching was also used for each database. Full paper publications and abstracts from January 1990 through February 2016 were considered for review.

Trial selection and patient population

Selection criteria included randomized controlled trials (RCT), prospective and retrospective studies. Non-human trials, case-control studies and publications in a language other than English were excluded. To reduce bias from learning curves of ESD and from possible patient selection, case series and studies with less than 50 patients were excluded. Studies reporting on ESD for non-gastrointestinal lesion (*e.g.* pharyngeal lesions), studies using hybrid ESD technique or studies targeting subepithelial lesions were excluded. We excluded studies with missing or unclear information on success rates of either *en bloc*, R0 or curative resections. Full-text articles of potentially relevant studies were obtained. Abstract publications containing our primary endpoint were included in the quantitative analysis.

Data extraction

Data were extracted by two authors independently (DCD, NS) and were then compared for accuracy. When data did not match, both reviewers reviewed the study a third time and divergences were resolved by consensus. In case of disagreement, a third reviewer (DvR) was available to arbitrate. The following information was collected: first author, journal, year of publication, number of patients, age and sex of patients, number of lesions, location and mean size of lesions, achievement of *en bloc*, R0 and curative resection, procedure time, type of knives used, procedure related bleeding and perforation as well as local recurrence.

Technical and oncologic outcomes of ESD

The primary outcome was efficacy of ESD based on information on either *en bloc*, R0 and curative resection rates. *En bloc* resection refers to a one-piece resection without fragmentation, R0 corresponds to a complete resection, which means no residual tumor. A resection was defined as curative when the histological findings showed no neoplasia in both lateral and vertical margins, as well as no lymphatic or venous invasion. The secondary outcomes included perforation, bleeding during or after the procedure, operation time and local recurrence rates. Perforation was either identified endoscopically post resection or by the presence of free air on imaging studies. We planned using the Clavien-Dindo scale for the analysis of procedural complications. In cases where data for number of males, lesion location, *en bloc*, R0, curative resection, or complications were expressed as a percentage, they were mathematically converted and rounded to the closest whole number. Eastern countries included studies reported from China, Japan, Korea and Taiwan. Western countries included studies reported from Europe, North and South America, as well as Australia. Each study's region was identified in the article or through the affiliation of the corresponding author.

Quality assessment and publication bias

Quality assessment of the included studies was done by identifying study designs as well as stratifying by study design according to their retrospective or prospective characteristic. We did not rely on GRADE or other tools because the data extracted for this review did not represent the primary outcome of all the included studies[12]. For the same reason we did not formally assess publication bias by means of funnel plots.

Statistical analysis

For each outcome of interest the number of outcomes over the number of lesions with available results for that outcome was expressed as a proportion. Those percentages were amalgamated and analyzed using the *metaprop* command of Stata 11 (StataCorp.2009. Stat Statistical Software: Release 11. College Station, TX: StatCorp LP). Meta-analyses were stratified by Eastern and Western study regions. Random effects meta-analysis was conducted for all outcomes, as heterogeneity was suspected *a priori* between Eastern and Western studies. Heterogeneity was assessed using the I-square statistic, with small *P*-values for I-square values indicating high chances of heterogeneity. To compare procedure time among Eastern and Western studies, a weighted average of these, as reported in minutes in the different studies, was computed.

Secondary analysis

We repeated analysis stratifying by organ (oesophagus, stomach, colo-rectum), by study design (retrospective, prospective) and by country.

RESULTS

The literature search identified 2532 studies (after duplicates were removed) (Figure 1). Based on title and abstract screening, 2159 studies were excluded. 135 further articles were excluded after full-text review of the publications. Thus, 238 studies were eligible for analysis, including 140 full-text and 98 abstract publications[6,13-249]. One study included ESD data from both a Japanese center and a center from the United States[18]. It was therefore divided into Western and Eastern part for our quantitative analysis, thus counting as two distinct studies. However, it was only considered as a single study when assessed for eligibility. The

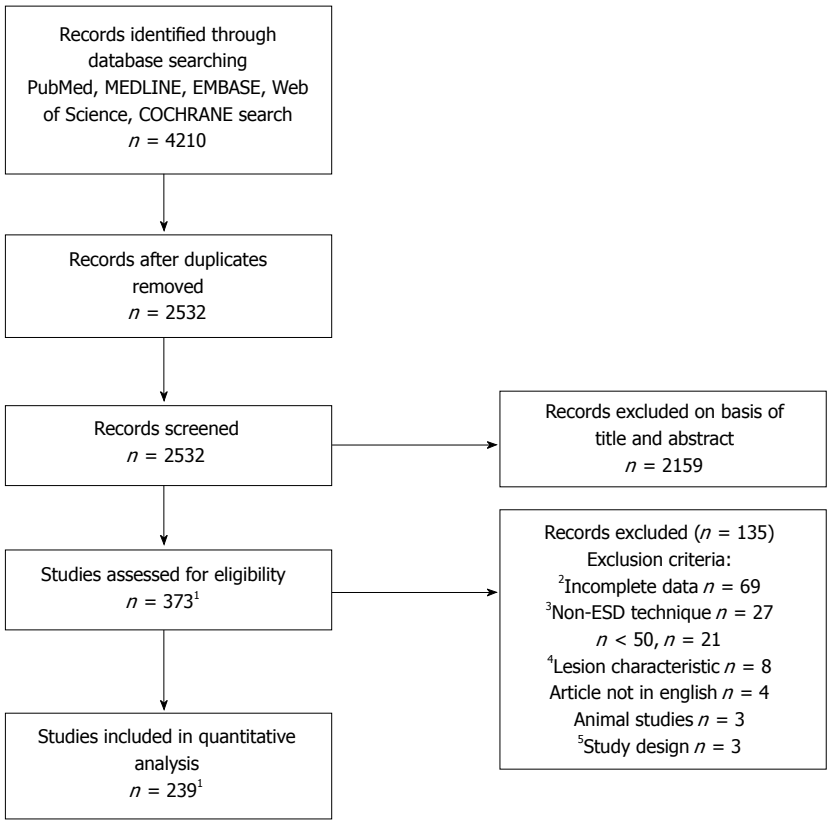

Figure 1 Flow diagram. The flow diagram shows the study selection process. [1]One study was divided into Western and Eastern parts for our quantitative analysis (thus counting as two studies). It was however only counted as a single study when assessed for eligibility; [2]Missing/incomplete data regarding our primary outcome (curative, *en bloc*, R0 resection); [3]Non-ESD study, hybrid ESD technique, ESD with snare; [4]Submucosal lesions, pharyngeal lesions; [5]Case-control, case report, questionnaire. ESD: Endoscopic submucosal dissection.

majority of the studies were retrospective cohort studies (168/239 trials). Only 7 studies were randomized controlled trials (although the randomized intervention was not ESD but some other aspect of endoscopy, such as, for example, analgesia type); others were prospective cohort studies. Overall, 90% of the included studies were from Eastern countries (216/239 trials) while only 10% (23 trials) were from Western countries (Belgium, Brazil, Colombia, France, Germany, Italy, Poland, Portugal, Turkey, and the United States).

A total of 84318 patients were enrolled in this analysis, including 82183 patients in the Eastern group and 2135 patients treated in Western countries. ESD was performed on 89512 lesions, comprising 87296 lesions in the Eastern group and 2216 in the Western group. Patient age and size of lesions were occasionally missing and the included studies were heterogeneous in terms of number of patients and lesions. The weighted average of subjects age was 66.4 and 66 years in the Eastern and Western group respectively. The weighted average for lesion size was 25.7 mm and 34.1 mm respectively. Table 1 and Supplementary Table 1 show characteristics of the included studies stratified into Eastern versus Western. Data regarding different knives used were not analyzed because most studies used multiple different knifes, and no standardization was available.

Curative resection rate

90 studies (81 Eastern, 9 Western) provided the curative resection rate involving 43854 lesions in the Eastern group and 922 lesions in the Western group. The overall curative resection percentage was 81.4% (95%CI: 79.6%-83.1%). This analysis showed a significant difference between the two groups in favor of the Eastern countries, where curative resection reached 82.3% (95%CI: 80.6%-84.1 %) compared to 71.3% (95%CI: 61.1%-80.5%) in Western countries. The results are shown as a forest plot in Figure 2A.

En bloc resection rate

215 studies (192 Eastern, 23 Western) collected the *en bloc* resection rate including 74883 lesions in the Eastern group and 2216 lesions in the Western group. The overall *en bloc* resection percentage was 94.4% (95%CI: 93.7%-95.0%). As for the curative resection, the pooled proportion of *en bloc* resection in the Eastern countries, 95.1% (95%CI: 94.4%-95.7%), was significantly higher than in the Western countries, 85.3% (95%CI: 81.3%-89.0%) (Figure 2B).

R0 resection rate

Overall, 154 studies (135 Eastern, 19 Western) reported the R0 resection rate totaling 50540 lesions in the Eastern group and 1948 lesions in the Western

Table 1 Characteristics of the included studies and patients

	Total	Eastern countries	Western countries
Age (yr)[1]	66.4	66.4	66
Total number of patients (*n*)	84318	82183	2135
Total number of lesions (*n*)	89512	87296	2216
Esophagus	5597	5276	321
Stomach	59966	59173	793
Duodenum	15	8	7
Colorectum	23934	22839	1095
Lesion diameter (mm)[1]	26	25.7	34.1
Procedure time (min)[1]	78	77	110
Total number of studies	239	216	23
Randomized controlled trial	7	7	0
Prospective studies	61	53	11
Retrospective studies	168	156	12
Full-text	140	130	10
Abstract	99	86	13

[1]Weighted average, with number of patients in study used as weights.

group. The overall R0 resection percentage was 88.0% (95%CI: 86.7%-89.3%). The R0 resection rate of the Eastern countries was statistically superior. The pooled proportion of the Eastern group was 89.5% (95%CI: 88.3%-90.6%) while that of the Western group is 74.4% (95%CI: 67.3%-80.9%) (Figure 2C).

Complications

Two hundred and nine studies reported complications related to the procedure involving 65956 patients in the Eastern group and 1893 in the Western group. There was no statistical difference for the overall bleeding and perforation rate between the Eastern and Western world. The proportions of bleeding (early and late) were 2.85% (95%CI: 2.44%-3.28%) and 4.03% (95%CI: 2.61%-5.70%) in the Eastern and Western groups, respectively (Figure 3A). Similarly, the perforation rates were 3.11% (95%CI: 2.79%-3.46%) and 3.38% (95%CI: 1.83%-5.29%) respectively (Figure 3B). As for the perforations requiring surgery, the Eastern countries percentage was significantly lower than the Western countries; 0.01% (95%CI: 0%-0.05%) and 0.53% (95%CI: 0.10%-1.16%) respectively (Figure 3C). With regards to complication rates, we initially planned using the Clavien-Dindo scale for the analysis of procedural complications, but because reporting of complication consequences was lacking in many studies we could not analyze this outcome.

Local recurrence and procedure time

Local recurrence was reported in 149 studies (12 Eastern, 137 Western), including 40936 patients in the Eastern group and 1188 patients in the Western group. The overall local recurrence percentage was 0.74% (95%CI: 0.48%-1.05%). There was no statistical difference between both groups and the proportion of local recurrence was 0.69% (95%CI: 0.42%-1.00%) and 1.82% (95%CI: 0.84%-3.07%) in the Eastern and Western group respectively (Figure 4).

The Western group weighted average of procedure time (110 minutes) was longer than the Eastern countries (77 min) (Table 1).

Stratification by organ

Because ESD outcomes may vary according to the organ, studies were stratified by lesion location (oesophagus, stomach, colo-rectum). For the oesophagus only the pooled proportion of *en bloc* resection showed a significant difference in favor of the Eastern countries. As for gastric lesions, all outcomes were similar except for local recurrence which was superior in Western countries. Finally, for colorectal lesions, Eastern countries had better curative, *en bloc* and R0 resection rate (Supplementary Figures 1-9).

Stratification by country

Meta-analyses for primary outcomes were stratified by countries. Secondary outcomes were not stratified because of the limited data available. Pooled proportions for curative, *en bloc* and R0 resections were similar among all Eastern countries and Western countries (Supplementary Figures 10-15).

Quality assessment

Stratification by study design was not different from the pooled proportion. The same trend was seen when stratifying by retrospective *vs* prospective results (Supplementary Figures 16-22).

Heterogeneity assessment

There was evidence of statistical heterogeneity for curative, *en bloc* and R0 resection as well as for bleeding, perforation, perforation requiring surgery and local recurrences (Figures 2-4).

DISCUSSION

To the best of our knowledge, this meta-analysis is the

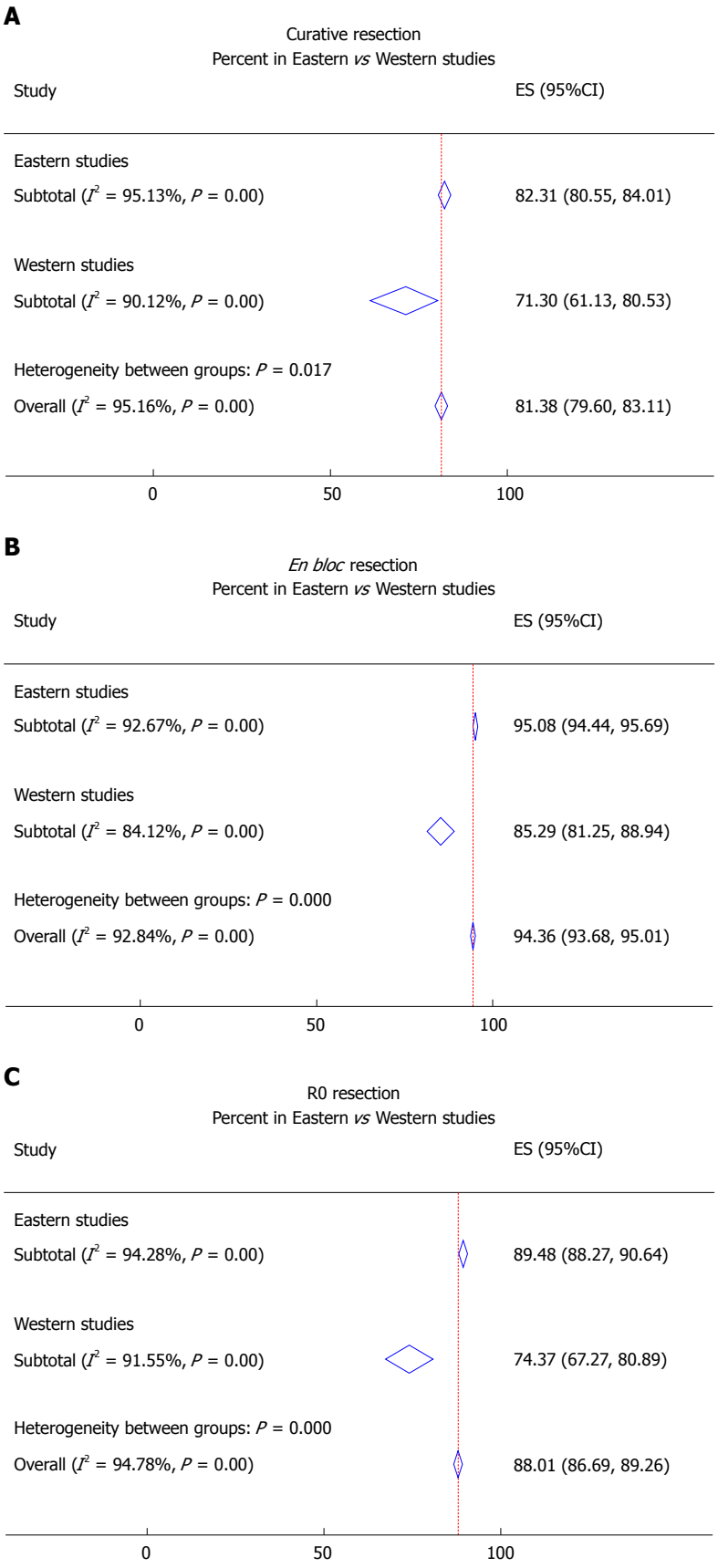

Figure 2 Efficacy of endoscopic submucosal dissection. Forest plot for curative resection (A), *en bloc* resection (B) and R0 resection (C).

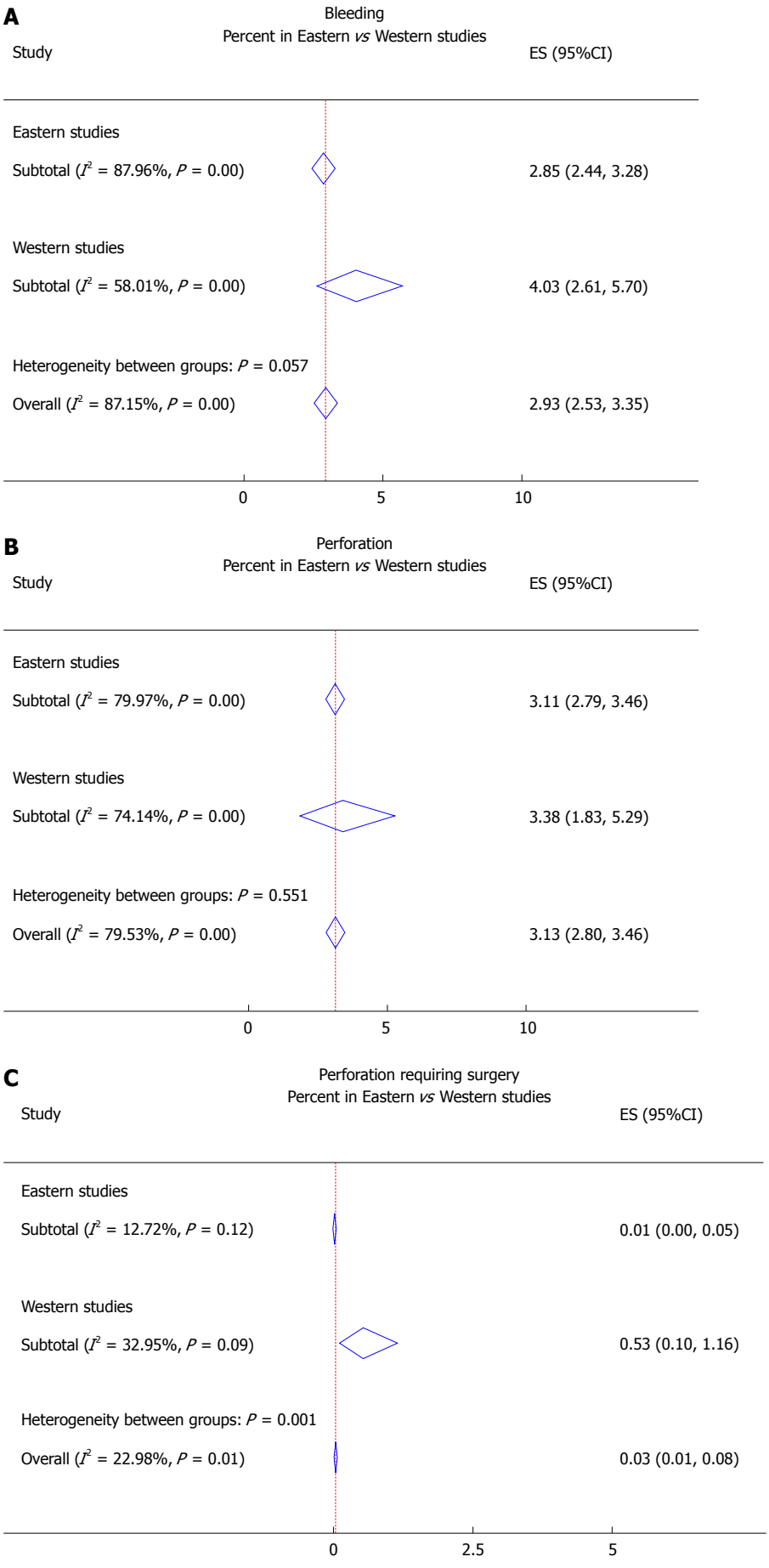

Figure 3 Complications of endoscopic submucosal dissection. Forest plot for bleeding (A), perforation (B) and perforation requiring surgery (C).

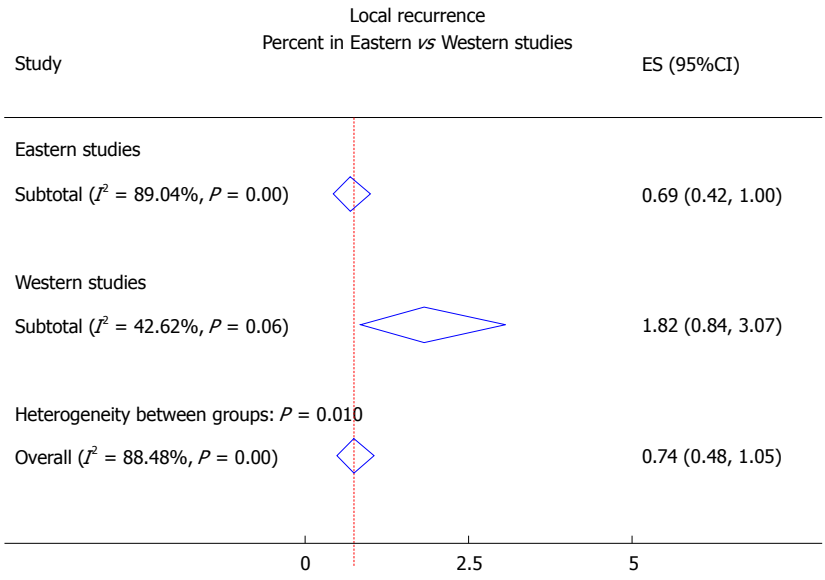

Figure 4 Forest plot for local recurrence.

first to compare ESD results between different regions of the world. Our results showed that Eastern countries have better rates of curative, *en bloc* and R0 resection compared to ESD results reported in North and South America, Europe and Australia. Moreover, ESD performed in Western countries was associated with a higher proportion of perforations requiring surgery. There was no significant difference found between regions with regards to other minor complications (*e.g.* bleeding) or local recurrence rates. Our meta-analysis also supports the fact that ESD efficacy varies according to the location of the lesion. Indeed, both Eastern and Western countries have similar outcomes for gastric lesions. As for colorectal ESD, which is a more difficult technique, the Eastern world shows better results. This difference could be due to the fact that Western countries still favor EMR for colic lesions and are less experienced. Furthermore, procedure times for ESD were longer in Western countries but the lesions removed by ESD in Western countries also tended to be larger.

The superiority of ESD compared to EMR with regard to curative, *en bloc* and R0 resection has been demonstrated in several meta-analysis[7-11]. However, these previous meta-analyses included studies only from the Eastern world thereby they do not provide an accurate representation for ESD outcomes in other regions of the world. The results from our meta-analysis showed that ESD outcomes differ globally and ESD results achieved for critical outcomes were different in the Western hemisphere. This might explain why the Western world is more reluctant to adopt widespread ESD use. However, there remains some level of uncertainty to which extent this is influenced by procedural expertise, case volumes and/or patient or lesion selection. With regards to regional complication rates, our analysis did not show any statistical differences in terms of overall perforation and bleeding rates, but we also found that ESD perforations requiring surgery occurred more often in Western countries. These results point out that the East has been doing these procedures longer than the West and therefore has more expertise with it. Furthermore, the overall numbers of lesions resected using ESD reflects well the steep difference in utilization (and thus likely expertise) with ESD in Eastern versus Western countries. Out of a total of 91582 ESD resection only 2289 ESDs were reported from the Western countries. It might therefore be important to consider available local expertise for clinical decision making and not assume that local outcomes will necessarily resemble outcomes from high volume Asian ESD centers. However, such expertise can be learned. A recent prospective multicenter French study reported outcomes for 314 patients undergoing ESD between 2010 and 2013 and 188 patients undergoing ESD between 2008 and 2010. An improvement in the rates of *en bloc* resection from 77.1% to 91.7% as well as a decrease in the complication rates (from 29.2% to 14.1% $P < 0.0001$) was demonstrated[250]. These rates are similar to outcomes reported from Japan, suggesting that adequate training, caseload and practice are mandatory to achieve optimal outcomes. However, at present few endoscopists in Western countries are adequately trained for ESD. Consequently, lesion size, lesion location and available expertise for ESD versus EMR need to be considered when choosing the appropriate approach for an individual patient. A recent literature review of the colorectal ESD series showed a low rate of superficially invasive cancer (8%) with a number needed to treat (NNT) for curative resection of 16. The majority of resected lesions were benign adenomas (82.2%) of which 26.8% were low grade dysplasia[251]. This study shows that histological description is a key element in evaluating ESD outcomes.

Data regarding the histology of the resected lesions was not analyzed in our meta-analysis because information was often incomplete or unavailable. We suggest that in future studies the histological features be described and analyzed.

Our meta-analysis has several limitations. First, there is an unequal distribution of studies between both groups. All the included RCTs are from the Eastern world, which also carries far more prospective studies than the Western world. Secondly, there is a lack of high quality studies, given that more than half of the included studies in this meta-analysis are retrospective. But in order to ensure the best snapshot of global ESD outcomes and include Western ESD outcomes, retrospective trial data needed to be included in our review. The large amount of retrospective studies and the differences of methodology in the prospective trials included are a limitation inherent to the current literature available. In order to avoid disadvantaging the Western group the inclusion of these publications was necessary. We propose that further studies be done in a more rigorous and standardized way. Because few studies reported systematic outcomes for trainee level, we decided to exclude studies with less than 50 patients to reduce bias from learning curves and patient selection. Future studies should report systematic outcomes for trainee level. Thirdly, there was evidence of statistical heterogeneity for most of our endpoints because of differences between study participants and differences of lesion size between the included studies. To reduce this heterogeneity, exclusion of studies contributing to the high heterogeneity could have been made. However, this was not done because it would have implied the exclusion of several Western studies that are already scarce. Fourthly, analyses were limited by missing data. Data regarding bleeding and use of anti-thrombotics were not analyzed because they were incomplete or unavailable in most studies. Such limitations seem to be an inherent weakness in the currently published ESD literature and a standardized and detailed reporting of ESD outcomes and use of anti-thrombotics seems warranted for future studies. However, our meta-analysis included 238 studies with 84318 patients (89512 ESD procedures) which reduces the above-mentioned risk for bias, thus optimizing generalizability.

In conclusion, this meta-analysis shows that ESD performed in Eastern countries is associated with better outcomes than studies reported from Western countries with regard to R0, *en bloc* and curative resection rates. Moreover, perforations requiring surgery are more common in Western studies. The clinical decision-making for or against ESD versus EMR should consider regional outcomes and locally available expertise as well as the necessity for resection according to oncologic standard based on the risk for cancer versus pre-cancerous lesions. Furthermore, standardized reporting of outcomes should be used for future ESD studies.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) has become the preferred approach to remove advanced gastrointestinal lesions in Asian countries while widespread adoption in the Western world remains limited.

Research motivation

Many previous meta-analyses suggest that ESD is a superior technique for treatment of precancerous gastrointestinal lesions or early cancers. However, there might be regional differences in outcomes since the majority of ESD publications come from the Eastern world. Studies evaluating differences for ESD outcomes between Eastern and Western countries are lacking.

Research objectives

To provide a global comparison of ESD outcomes between Eastern and Western countries.

Research methods

A systematic review and meta-analysis were conducted on studies reporting ESD outcomes. Were excluded studies with less than 50 patients, using hybrid ESD technique or targeting subepithelial lesions. Primary and secondary outcomes were efficacy of ESD (curative, *en bloc* and R0 resection), complications (bleeding, perforation) and other related factors as local recurrence and procedural time.

Research results

Our meta-analysis showed that Eastern countries have better ESD outcomes compared to Western countries for curative, *en bloc* and R0 resection. ESD performed in Western countries were associated with a higher proportion of perforation requiring surgery. Subgroups analysis by organ showed similar outcomes for gastric lesions while Eastern countries had better curative, *en bloc* and R0 resection rates for colorectal lesions.

Research conclusions

This meta-analysis provided evidence that there are regional differences for ESD outcomes. Eastern countries show better ESD outcomes compared to Western countries.

Research perpective

Clinical decision-making for or against ESD should consider such outcomes and locally available expertise. Standardized reporting of outcomes should be used for future ESD studies.

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